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EFFECTS OF SUPPLEMENTAL OSTEOPONTIN ON INTESTINAL DEVELOPMENT AND  
SERUM ANTIBODY RESPONSES TO ROTAVIRUS VACCINATION IN PIGLETS

BY

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THESIS

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## Abstract

Milk contains numerous bioactive substances including immunoglobulins, cytokines, growth factors and components that exert antibiotic and prebiotic activity (Field, 2005). Little is known about the biological effects of individual milk bioactives, despite the fact that natural milk improves intestinal development and immune system functions in neonates (Donovan et al., 1994; Field, 2005) relative to milk formula. Characterization of the biological effects of such components is important for optimal production of infant milk formulas to be used when mother's milk is not available. Milk components with preliminary evidence of positive effects on the intestinal growth and mucosal immunity include osteopontin (OPN). Osteopontin is a phosphorylated acidic glycoprotein expressed by a number of different immune and non-immune cells and tissues (Sodek et al., 2000). It is also present in body fluids including blood, bile and milk (Sodek et al., 2000). Osteopontin is a multifunctional protein that is implicated in a wide number of biological processes including cell survival, bone remodeling, and immune modulatory functions (Sodek et al., 2000). Furthermore, Schack and colleagues (2009) demonstrated that the concentration of OPN in human milk is considerably higher than in bovine milk and infant formulas. Taken together, it is likely that OPN plays a role in the early development of gastrointestinal tract and mucosal immune responses in infants. Since the neonatal pig shares anatomical, physiological, immunological, and metabolic similarities with the human infants (Moughan, et al., 1992), they were selected as the animal model in our studies.

Our first aim was to investigate the effects of OPN on piglet intestinal development. Newborn, colostrum-deprived piglets (n=27) were randomized to receive three treatments: formula with bovine OPN (**OPN**; 140 mg/L); formula alone (**FF**); or sow reared (**SR**) for 21

days. Body weight, intestinal weight and length, mucosal protein and DNA content, disaccharidase activity, villus morphology, and crypt cell proliferation were measured. Statistical significance was assigned at  $P < 0.05$ . No significant effects of OPN were observed for body weight, intestinal weight and length. Mucosal protein content of SR piglets was lower than FF and OPN piglets in the duodenum, but higher than FF and OPN piglets in the ileum. No significant effects of diet in mucosal DNA content were detected for the three regions of the small intestine. Lactase and sucrase activities of SR piglets were higher than the two formula-fed groups in the duodenum, lower in the ileum. No significant effects of diet on lactase and sucrase activities were noted between two formula-fed groups in the duodenum and ileum. Jejunal lactase activity of FF piglets was higher than SR piglets, whereas no significant effect of diet was observed in jejunal sucrase activity among the three groups. Duodenal and ileal villus height and villus area of SR piglets were lower than two formula-fed groups, while OPN piglets did not differ from FF piglets. There was a significant effect of diet ( $P < 0.0001$ ) on jejunal crypt cell proliferation, with proliferation in OPN piglets being intermediate between that of FF and SR. In summary, supplemental OPN increased jejunal crypt cell proliferation, independent of evident morphological growth, and had a minor impact on disaccharidase activity in the small intestine of neonatal piglets.

Rotavirus (RV) is the most common viral cause of severe gastroenteritis in infants and young children worldwide (Parashar et al., 2006). Maeno et al. (2009) reported that OPN knockout (OPN-KO) suckling mice were more susceptible to RV infection compared to wild-type (WT) suckling mice. To detect the role of OPN in intestinal immune responses of neonates, the goal of the second study was to evaluate whether supplemental OPN influenced the serum antibody responses to RV vaccination in neonatal piglets. Newborn, colostrum-deprived piglets

were randomized into two dietary groups: formula with bovine OPN (**OPN**; 140 mg/L) and formula alone (**FF**) for 35 days. On d7, piglets in each dietary group were further randomized to receive rotavirus (RV) vaccination (Rotarix<sup>®</sup>) (FF+RV and OPN+RV) or remained non-vaccinated (FF+NV and OPN+NV). Booster vaccination was provided on d14. Blood samples were collected on d7, 14, 21, 28 and 35. RV-specific serum immunoglobulin (Ig) G, IgA, IgM and total serum IgG, IgA, IgM were measured by ELISA. Statistical significance was assigned at  $P < 0.05$ , with trends reported as  $P < 0.10$ . Body weight gain was unaffected by diet and/or vaccination. No significant effect of oral OPN supplementation was observed for RV-specific antibody responses and total Igs levels. After the combination of dietary groups, RV piglets had significantly higher RV-specific IgM concentrations compared to NV piglets. Although there were higher means of RV-specific IgG and RV-specific IgA concentrations in RV group than their counterparts in NV group, the difference did not reach statistical significance. RV-specific IgM reached a peak at d7 post booster vaccination (PBV), whereas the RV-specific IgG and IgA peaked later at PBV 14 or 21. Total Igs were unaffected by RV vaccination but were significantly increased over time, following similar pattern as RV-specific Igs. In summary, neonatal piglets generated weak antibody responses to RV vaccination. Supplemental OPN did not enhance RV-specific serum antibody responses and total serum Igs levels in neonatal piglets with or without RV vaccination.

In conclusion, we observed normal developmental changes in the small intestine and serum Igs levels in neonatal piglets over time. Oral OPN supplementation showed minimal impacts on intestinal development and no effect on serum Igs levels. The role of supplemental OPN on the growth and development of infants is still inconclusive. Future studies should

measure other physiological and immunological parameters by using different models of vaccination or infection.

*To my Family and Friends*

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## **List of Abbreviations**

ASC	Antibody secreting cell
AttHRV	Attenuated, human rotavirus
BCG	Bacillus Calmette-Guerin
BSA	Bovine serum albumin
BW	Body Weight
d	Day
DAPI	4', 6-diamidino-2-phenylindole dihydrochloride
DC	Dendritic cell
EdU	5-ethynyl-2'-deoxyuridine
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunosorbent spot
FF	Formula-fed
Gnotobiotic	Gn
hr	hour
HRP	Horseradish peroxidase
ISCOM	Immunostimulating complex
IFN- $\alpha$	Interferon-alpha
IFN- $\gamma$	Interferon-gamma
Ig	Immunoglobulin
IL	Interleukin
LoMatAb	Low-titer maternal antibodies
LP	Lamina propria

LPH	Lactase-phlorizin hydrolase
LPMNC	lamina propria mononuclear cells
LPS	Lipopolysaccharide
MatAb	Maternal antibodies
min	minute
NF- $\kappa$ B	Nuclear factor-Kappa B
NK	Natural killer
NO	Nitric oxide
NSP	Nonstructural proteins
NV	Non-vaccinated
OPN	Osteopontin
PBV	Post booster vaccination
RGD	Arginine-glycine-aspartate
RV	Rotavirus
SPP	Secreted phosphoprotein
SR	Sow-reared
TGF- $\beta$	Transforming growth factor $\beta$
Th cell	T helper cell
TLR	Toll-like receptor
V	Vaccinated
VLP	Virus-like particles
VP	Viral structural protein
TNF- $\alpha$	Tumor necrosis factor-alpha

# Chapter 1

## Literature Review

### 1.1 Osteopontin

Osteopontin (OPN) was first described as a secreted, 60-kDa transformation-specific phosphoprotein (Senger et al.1979) and subsequently was rediscovered by molecular cloning of the transformation-associated gene 2ar (Craig et al., 1989). The name “osteopontin” was introduced to reflect the potential of the bone protein to serve as a bridge between cells and hydroxyapatite through RGD and polyaspartic acid motifs discovered in the primary sequence of the protein (Oldberg et al., 1986). However, the same gene product was identified as a putative lymphokine produced by activated lymphocytes and macrophages and called Eta-1 or early T-lymphocyte activation gene I (Palarca et al., 1989), and, thus, a more general pattern of expression for OPN was emerging. Accordingly, secreted phosphoprotein (SPP 1) was introduced as an alternate name, to reflect a broader functional role of this protein.

### Structural Characteristics

The functional motifs of the OPN molecule, illustrated in **Figure 1.1** (Wang & Denhardt, 2008), may provide clues to OPN’s broad biological functions. OPN is expressed by a single-copy gene, in a cluster of SIBLING (Small Integrin-Binding Ligand, N-linked Glycoprotein) family proteins, as a ~34 kDa nascent protein that is extensively modified by posttranslational events. The human gene contains 7 exons, spans ~11.1 kb, and maps to the long arm of chromosome 4 (4q13) (Young et al., 1990). In comparison, the ~4.8 kb mouse gene is at the locus of the Rickettsia resistance gene Ric<sup>r</sup> on chromosome 5 (Miyazaki et al., 1989), and the pig gene is on chromosome 8 (Denhardt and Guo, 1993).

The expression of OPN is affected by a large number of hormones, cytokines, and growth factors that can influence the rate of gene transcription, mRNA processing, stability and translation, as well as post-translational modifications. Increased expression of OPN is frequently associated with an increase in transcription of the OPN gene, which is regulated by transactivation of cis-acting elements in the gene promoter. Its promoter is responsive to a number of different transcription factors (Denhardt et al., 2003). Pro-inflammatory cytokines stimulate OPN gene transcription and expression. For example, activation of macrophages with lipopolysaccharide (LPS) and nitric oxide (NO) induces OPN gene expression and protein secretion (Mazzali et al., 2002). Classical mediators of acute inflammation such as tumor necrosis factor alpha (TNF- $\alpha$ ) and interleukin-1beta (IL-1 $\beta$ ) strongly induce OPN expression (Patarca et al., 1993). Additional factors that can up-regulate OPN expression include angiotensin II, transforming growth factor-beta (TGF- $\beta$ ), hyperglycemia and hypoxia (Ricardo et al., 2000; Hullinger et al., 2001; Sodhi et al., 2001).

Full-length OPN is composed of about 300 amino acids (297 in mouse; 314 in human); there are also functionally important cleavage products and occasional splice variants. Although expressed as a ~34 kDa nascent protein, extensive posttranslational modifications increase its apparent molecular weight to about 44 kDa; in SDS-PAGE gels it migrates in the range of 50~75 kDa depending on conditions (Sodek et al., 2000). Both highly conserved sequence motifs and post-translational modifications contribute to different functional activities of OPN (Kazanecki et al., 2007).

The protein is rich in aspartic and glutamic acid and serine residues, and contains a polyaspartic acid motif, through which the protein can bind to hydroxyapatite and calcium ions, and an arginine-glycine-aspartate (RGD) sequence that can mediate cell attachment. In addition,

multiple sites of Ser and Thr phosphorylation and sites of both N- and O-linked glycosylation exist, together with a thrombin cleavage site. Variations in phosphorylation, glycosylation, and sulphation generate different functional forms of OPN which may be found in the same or different tissues.

### **Amino Acid sequence comparisons**

The amino acid sequences of OPNs derived from human (Kiefer et al., 1989), cow (Kerr et al., 1991), pig (Wrana et al., 1989), rabbit (Tezuka et al., 1992), rat (Oldberg et al., 1986), mouse (Craig et al., 1989), and chicken (Moore et al., 1991 ) cDNAs are currently available. Comparison of the mammalian OPN sequences reveals a high conservation in the amino- and carboxy-terminal regions and in the polyaspartate segment, as well as in the GRGDS and thrombin cleavage sites and in several potential phosphorylation sites (**Figure. 1.2**). Of the 333 amino acids in the consensus sequence for mammalian OPNs (CLUSTAL W program, Thompson et al., 1994), 107 amino acids are identical, with 59 amino acids retaining high similarity and a further 26 with lower similarity, for a conservation of 58% of the amino acids. Notably, there is also high identity (10/21 residues identical) in a sequence (220-240) that is missing in bovine OPN and in a short sequence (312-316) that is missing in both the cow and the pig (Sodek et al., 2000). When the avian sequence is included in the comparison, the number of perfect matches is reduced considerably, to 52, with 48 amino acids retaining high similarity and 37 with lower similarity, for a conservation of 39% (Sodek et al., 2000). Despite the reduced conservation, the GRGDS motif, the polyaspartic acid sequence, and the thrombin cleavage sites are retained, together with several phosphorylation sites (Sodek et al., 2000). Nevertheless,

distinct differences in the developmental expression have been reported which could reflect functional differences between chicken and mammalian OPNs (Thayer and Schoenwolf, 1998).

### **Structure-Function Analysis**

Cell Attachment and Signaling through Integrins. As shown in **Figure 1.1** (Wang & Denhardt, 2008), located near the center of the OPN protein is a RGD domain, a motif common to many extracellular matrix proteins and known to engage many integrins. The protein also contains an aspartate-rich region, two heparin-binding sites, a thrombin cleavage site and a region near the C-terminus that binds specific CD44 variants. Via the RGD sequence, OPN interacts with a variety of cell surface receptors, including the  $\alpha_v\beta_1$ ,  $\alpha_v\beta_3$ ,  $\alpha_v\beta_5$ ,  $\alpha_v\beta_6$ , and  $\alpha_5\beta_1$  (O'Regan & Berman, 2000; Yokosaki et al., 2005). A cryptic integrin binding site is exposed after thrombin cleavage between the L and R residues; it is important in the regulation of cell adhesion, spreading and migration by promoting the adherence of cells expressing  $\alpha_4$  and  $\alpha_9$  integrins ( $\alpha_9\beta_1$ ,  $\alpha_4\beta_7$ ), which are preferentially expressed by leukocytes (Helluin et al., 2000). Both intact OPN and the N-terminal fragment of OPN promote leukocyte adhesion to  $\alpha_4\beta_1$ ; interestingly, there are two different binding sites for  $\alpha_4\beta_1$  present in a 38-amino acid domain within the N terminal thrombin fragment (Bayless et al., 1998). The interaction of OPN with the  $\alpha_8\beta_1$  integrin is necessary for normal kidney morphogenesis (Denda et al., 1998).

Cell Attachment and Signaling through CD44. OPN interacts with CD44v6- and v7-containing isoforms, stimulating (in a human tumor cell) transcription of the CD44 gene and also the abundance of CD44s, v6 and v9 at the cell surface (Khan et al., 2005). The interaction of OPN with CD44 appears to be RGD-independent and to require the presence of  $\beta_1$  integrins (Katagiri et al., 1999). The ligation of CD44 variant isoforms by OPN mediates chemotaxis and

adhesion of fibroblasts, T cells and bone marrow cells (Denhardt et al., 2001; Wai et al., 2004). CD44 engagement with OPN down-regulates IL-10 expression in peritoneal macrophages (Ashkar et al., 2000). OPN promotes proliferation and survival of IL-3-dependent bone marrow cells; anti-CD44 antibody attenuates these actions (Lin et al., 2000). A C-terminal location has also been suggested as the site of CD44 interaction (He et al., 2006). A monoclonal antibody recognizing the C-terminal region of OPN blocked the ability of OPN to attach to cells, raising the possibility that attachment of OPN to CD44 modulates the cells' ability to bind to the RGD binding motif via the RGD sequence (Kazanecki et al., 2007). Kazanecki et al. (2007) have hypothesized that a sequence in the C-terminal region forms a  $\beta$ -sheet structure with the RGDSVVYLGR domain, thereby interfering with the RGD-integrin interaction.

### **Post-translational Modifications (PTM) of Osteopontin**

The PTM of OPN influences its function (Kazanecki et al., 2007). The OPN protein is highly modified, including Ser/Thr phosphorylation, O-linked glycosylation, tyrosine sulfation and sialylation. Many sites of PTMs are conserved across species; however, the degree of modification of the protein varies depending on the source tissue and cell type or differentiation stage. For example, both bovine and human milk OPN have a large number of phosphorylated serine residues (28 and 32, respectively, mostly in motifs implicating casein kinase 2). These are located in clusters (**Figure 1.1**, Wang & Denhardt, 2008) that are distant from the RGDSVVY and glycosylated regions (Christensen et al., 2005, 2007). In contrast, rat bone OPN contains only 10-11 phosphorylated residues (Keykhosravani et al., 2005). Phosphorylation of OPN appears necessary for various physiological functions, including migration of cancer cells (Al-Shami et al., 2005), adhesion and bone resorption by osteoclasts (Razzouk et al., 2002),



inhibition of smooth muscle cell calcification (Jono et al., 2000) and regulation of mineralization (Gericke et al., 2005). The phosphorylation of OPN is usually heterogeneous, and it is not known whether certain specific sites are critical for a given function.

### **Tissue Distribution**

Osteopontin is expressed in a variety of tissues, such as bone, kidney, brain, vascular tissues, brain cells and specialized epithelia found distal renal tubules and in the gut, as well as in activated macrophages and lymphocytes (Sodek et al., 2000). Osteopontin also appears in biological fluids, including blood, milk, urine and seminal fluid (Sodek et al., 2000). The amount of OPN in normal plasma of women ranges from 22 to 122  $\mu\text{g/L}$ , with a median level of 47  $\mu\text{g/L}$  (Singhal et al., 1997), whereas in urine, OPN ranges from 1.9 to 4.3  $\mu\text{g/mL}$ , with  $\sim 4$  mg excreted per day (Min et al., 1998). In milk, OPN is present as an intact protein or in several proteolytically generated N- and C-terminal fragments (Sørensen et al., 2003).

Osteopontin is found in a restricted distribution in healthy adult humans and animals, whereas studies of OPN in disease and injury have revealed striking up-regulation under conditions of inflammation and tissue remodeling (Giachelli et al., 2000). Thus, OPN synthesis is induced in smooth-muscle cells and cardio-myocytes in cardiovascular diseases, including atherosclerosis (Giachelli et al., 1993) and ventricular hypertrophy (Graf et al., 1997). Similarly, OPN is induced in kidney diseases, such as interstitial nephritis (Sibalic et al., 1997) and in pathological mineralization such as kidney stone formation (McKee et al., 1995). Synthesis of OPN by activated lymphocytes and macrophages (Patarca et al., 1989, 1993) can account for the presence of OPN in diseased and damaged tissues. Studies on granulomatous inflammation have indicated that, as well as acting as a chemoattractant for T-cells, OPN can promote adhesion of

T-cells and possibly amplify a CD3-mediated proliferative response (O'Regan et al., 1999). The increased OPN activity following thrombin digestion observed in these studies suggests a mechanism whereby OPN and thrombin can modulate T-cell recruitment and activation. All of these situations have in common an inflammatory component, leading investigators logically to study the potential role of osteopontin in host defense responses.

### **Signaling Pathways of Osteopontin**

As depicted in in **Figure 1.3** (Wang & Denhardt, 2008), OPN can signal through several different pathways to affect gene transcription. OPN signaling through integrins can modulate (via activation of Ras and Src) the phosphorylation of kinases (NIK, IKK $\beta$ ) involved in nuclear factor kappa-B (NF $\kappa$ B) activation (Rice et al., 2006; Scatena et al., 1998); this results in the degradation of I $\kappa$ B, an inhibitor of NF $\kappa$ B (Vejda et al., 2005). NF $\kappa$ B regulates expression of many inflammatory cytokines. Consequently, OPN may modulate immune responses through activation of the NF $\kappa$ B pathway, and the absence of OPN may be associated with diminished cytokine functionality. In a study of the role of OPN in the exacerbation of experimental autoimmune encephalomyelitis, it was observed that OPN appeared to enhance the survival of myelin-reactive T cells through the regulation of the transcription factors Foxo3a and NF $\kappa$ B and the expression of genes involve in apoptosis (Hur et al., 2007).

In addition, OPN signaling through CD44 engagement promotes cell survival by activating the PI3K/Akt pathway (Lin et al., 2001). A genetic profiling study documented that OPN is a downstream effector of the PI3K/Akt pathway, which is antagonized by phosphatase and tensin homolog (PTEN); melanoma lines defective in PTEN expression exhibited increased OPN expression (Packer et al., 2006). Lastly, in the presence of viral infections, intracellular

OPN is found to be localized together with the MyD88 and TLR9 complex near the inner cytoplasmic membrane; it activates nuclear translocation of transcription factor IRF7 to induce robust IFN- $\alpha$  production (Shinohara et al., 2006).

### **Osteopontin and Innate Immunity**

Most infectious agents induce inflammatory responses by activating innate immunity in processes often involving macrophages and neutrophils. These cells are the professional phagocytes that provide the first line of defense of the immune system. Epithelial damage causes release of the cytokines IL-1 and IL-8, which recruit macrophages and neutrophils to the site of injury (Wang et al., 2008). Macrophages express toll like receptors that recognize pathogen-associated molecular patterns (PAMPs) and engulf the pathogen, leading to the secretion of cytokines/chemokines that attract cells such as neutrophils and monocytes to sites of injury or infection (Wang et al., 2008). The role of OPN in innate immunity is reflected in its protective function in infectious diseases. It contributes to the mucosal defense against viral pathogens. Significant up-regulation of OPN mRNA in murine intestine and epithelial cells was observed in rotavirus infection, and the disease was protracted in OPN-deficient mice (Rollo et al. 2005). Likewise, OPN deficient mice are impaired in their ability to clear *Listeria monocytogenes* after systemic infection (Ashkar et al., 2000). **Figure 1.4** (Wang & Denhardt, 2008) illustrates aspects of the involvement of OPN in innate and adaptive immune functions.

### **Osteopontin and Macrophages**

Monocytes express a low level of OPN, but as they differentiate into macrophages OPN expression is increased; OPN becomes constitutively expressed in macrophages and can be

further up-regulated by LPS stimulation (Gao et al., 2004). In addition, OPN has been shown to regulate macrophage functions including migration (Zhu et al., 2004), activation (Rollo et al., 1996), phagocytosis (McKee & Nanci, 1996), proinflammatory cytokine production (Koguchi et al., 2002) and nitric oxide synthesis (Scott et al., 1998) in response to various inflammatory challenges. In the absence of OPN expression, macrophage migration and cytokine production are impaired (Sodek et al., 2006). At sites of tissue injury, OPN produced by macrophages fosters cell adhesion and may act as an opsonin facilitating phagocytosis of foreign bodies (McKee & Nanci, 1996). The interaction of OPN with integrins and CD44 (v) stimulates via different signal transduction pathways distinct patterns of cytokine/ chemokine expression and the specific immune response (Wang & Denhardt, 2008).

### **Osteopontin and Dendritic Cells**

Dendritic Cells (DCs) are a type of unique immune cell population that functions as a link between innate and adaptive immunity. They function as effector cells in the innate immune system to provide the first line of defense by non-specifically recognizing invading pathogens. Through maturation and migration from the sites of infection towards secondary lymphoid organs, they turn into professional antigen-presenting cells to induce antigen specific T cell activation. Osteopontin is highly expressed in immature DCs, but its production decreases during maturation (Kawamura et al., 2005). As observed for other inflammatory cells, OPN is also a survival factor for DCs (Kawamura et al., 2005). Osteopontin-activated DCs produce IL-12 and TNF- $\alpha$ , and, when incubated with naive T cells, can induce naive T cells into Th1-polarizing, interferon-gamma (IFN- $\gamma$ )-producing T cells (Renkl et al., 2005). Thus, OPN's presence in T cell-DC interactions may decisively influence T-cell polarization. This concept is emphasized by

the deficiency of Th1 immunity in OPN deficient mice, which leads to a compromised host defense against bacterial and viral infections (Rollo et al., 2005) and ameliorated autoimmunity (Chabas et al., 2001).

### **Osteopontin and Cell-Mediated Immunity**

Osteopontin is considered to be an important lymphocyte mediator that is secreted by activated T-lymphocytes. It induces macrophage migration and suppresses the production of reactive oxygen species, while enhancing immunoglobulin production and proliferation of B-lymphocytes (Weber & Cantor, 1996). Osteopontin was shown to be required for cell-mediated immunity and the development of the Th1 pathway and macrophage activity (Ashkar et al., 2000).

Elevated OPN expression is found in various immunological disorders. Up-regulation of OPN is implicated in the formation of granulomas in tuberculosis, sarcoidosis and silicosis, all of which involve cell-mediated immune reactions (Nau et al., 1997). Studies of OPN null ( $OPN^{-/-}$ ) mice revealed that OPN's main immune-modulatory function is the enhancement of Th1 over Th2 immunity through two general mechanisms: regulation of cytokine production via 1) interaction with the  $\alpha_v\beta_3$  integrin to induce pro-inflammatory IL-12, and 2) ligation of CD44v to suppress anti-inflammatory cytokine IL-10 production (Ashkar et al., 2000). Autocrine amplification of cytokine production and cross-inhibition ensure progressive polarization of the T-helper cells towards either Th1 or Th2 cells (Abbas et al., 1996).

Osteopontin is produced by activated T cells and is classified as a Th1 cytokine because it modulates cell-mediated immunity by promoting the Th1 response. In T-helper (CD4+) T-cells, OPN mRNA is expressed in Th1 polarized cells (Nagai et al., 2001). Moreover, soluble OPN

may modulate the differentiation and proliferation of CD4<sup>+</sup> and CD8<sup>+</sup> (cytotoxic) T cells (Higuchi et al., 2004). OPN can also stimulate human peripheral blood T cells to express IFN- $\gamma$  and CD40L, which subsequently induce IL-12 expression from monocytes; up-regulation of CD40L by OPN in T-lymphocytes (O'Regan and Berman, 2000) provides mechanistic support for the association of OPN with polyclonal B-cell proliferation and humoral autoimmune disease (Weber et al., 1996). Furthermore, the increase of OPN levels was inversely correlated with in vitro T-cell IL-10 production. Osteopontin regulates cell-mediated immunity by promoting T cell proliferation and migration as demonstrated in transgenic mice over-expressing OPN (Wang & Denhardt, 2008). While OPN is generally classified as a pro-inflammatory cytokine, it appears to have anti-inflammatory effects in certain pathological contexts. The multifunctional nature of OPN may reflect expression of specific isoforms, levels or timing of production and tissue distribution (Wang & Denhardt, 2008). Dual (pro- vs. anti-inflammatory) effects of OPN in intestinal inflammation have been reported. During the acute phase of colitis, OPN seems to activate innate immunity, reduce tissue damage and initiate, mucosal repair whereas during chronic phase it promotes the Th1 response and promotes inflammation (Heilmann et al., 2009).

### **Osteopontin and Mucosal Protection**

Determinants of Mucosal Immunity. A specialized epithelial barrier lining the oral cavity, the intestinal tract, respiratory tract, and urogenital systems provides the primary protection for mucosal tissues against pathogens and other environmental insults. Breakdown of the barrier function leads to the infiltration of bacteria or luminal noxious agents that cause inflammatory diseases, including periodontal disease in the oral cavity, inflammatory bowel diseases (IBDs) (Tlaskalova-Hogenova et al., 2004), respiratory diseases (Delclaux & Azoulay, 2003) and

urogenital diseases (Mulvey, 2002). Physical protection is achieved by the formation of tight junctions connecting the epithelial cells, while goblet and other specialized secretory cells produce mucous enriched with antimicrobial glycoproteins, IgA class antibodies, cytokines, and chemokines to defend against the invasion of pathogens and maintain the integrity of the epithelial barrier (Acheson & Luccioli, 2004). Another aspect of this barrier is the viscosity of the mucous secreted by the epithelial cells, which can prevent the adherence of particles or micro-organisms that are otherwise expelled by ciliary movement in the respiratory tract, or by peristalsis in the gut. Failure of these barrier functions may lead to recurrent respiratory tract infections or infestation and infection of the gut lumen, respectively (Sodek et al., 2006). Noxious irritation/damage of the epithelial barrier can be mediated by epithelial cells through special recognition receptors such as death Toll-like receptors, by specialized epithelial cells (microfold; M-cells), or, more directly, by the penetration of bacteria or their products (Shi & Walker, 2004). Penetration of the epithelial barriers results in activation of resident neutrophils (polymorphonuclear leukocytes; PMNs) and macrophages; these are professional phagocytes, which provide the "immediate innate immune response" and non-specifically engulf foreign material and bacteria (**Figure 1.5A**, Sodek et al., 2006). Cytokines and chemokines released from the injured epithelium as well as the leukocytes increase neutrophil and macrophage infiltration into the tissue, and thereby initiate the inflammatory response (Sodek et al., 2006). Long-term residents of the subepithelial tissue, the antigen-presenting cells (APC)/dendritic cells, which are specialized macrophages, initiate the more specific "adaptive immune responses". The adaptive system is based on a specific recognition between APC and naive T-cells, resulting in the differentiation of Th1, T-helper, and cytotoxic (NKT) T-cells, which control the immune reaction (**Figure 1.5B**, Sodek et al., 2006). Notably, many studies have correlated OPN

expression with epithelial barrier changes (Gassler et al., 2002), with macrophage (O'Regan et al., 2001; Zhu et al., 2004), neutrophil (Alstergren et al., 2004), and lymphocyte activities (Ashkar et al., 2000), and with the function of reparative fibroblasts (Sodek et al., 2002).

Osteopontin and the Epithelial Barrier Function. Osteopontin has been recognized as an important luminal regulator (Brown et al., 1992), due to its expression by epithelial cells covering luminal cavities capable of active secretion and absorption of nutrients or gasses. Indeed, earlier studies showing that epithelial cells secrete OPN first indicated that OPN is involved in controlling epithelial barrier permeability and secretory functions (Butler, 1989). In the gastrointestinal tract, a layer of columnar epithelial cells separates the underlying mucosa from the lumen and provides a reservoir for macrophages and T- and B-lymphocytes, which are concentrated in an organized subepithelial network along the gut and can be identified in focal areas, such as the Peyer's patches in the distal small intestine (Sodek et al., 2006). Moreover, the epithelial layer also contains specialized cells, such as the microfold (M) cells, capable of recognizing specific bacteria and antigens and transferring them to specialized APCs that reside in the vicinity of the epithelial layer, and take part in the initiation of the adaptive defense process (Mowat, 2003; Acheson & Luccioli, 2004) (**Figure 1.5**, Sodek et al., 2006). The MHC class-II and Toll like receptors on specialized epithelial cells may be involved directly with antigen presentation to underlying CD4<sup>+</sup> T-cells (Acheson & Luccioli, 2004) (**Figure 1.5**, Sodek et al., 2006). It has been suggested that the constitutive expression of OPN by epithelial cells is required for maintaining the epithelial barrier in the intestines (Gassler et al., 2002). OPN also has the potential to regulate specific functions of epithelial cells involved in the barrier defense process (**Figure 1.5**, Sodek et al., 2006), such as the expression of MHC-II and Toll receptors,



which aid in antigen presentation and inflammatory signaling (Mowat, 2003; Iwasaki & Medzhitov, 2004).

The ability of the epithelial barrier to resist stress and trauma, and to regenerate, is important for the subsequent repair of the diseased tissues. Death of epithelial cells, therefore, is an important event associated with mucosal damage (Hagimoto et al., 2002; Kruidenier et al., 2003). In view of the role of OPN in cell survival, its presence may be important for supporting programmed cell death and preventing rapid necrotic death, which can result in intense inflammation and loss of epithelial barrier and protection (Sodek et al., 2006).

### **Osteopontin and Biomineralization**

As mentioned earlier, OPN is highly expressed in mineralized tissues including bone and teeth. In fact, it is one of the most abundant non-collagenous proteins in bone (Giachelli & Steitz 2000). Furthermore, OPN is also invariably found in pathological calcifications of soft tissues. The association of OPN with biomineralization has led to a great number of studies aimed at elucidating its function in this process. At least three different functions of OPN in calcified tissues have been proposed based on in vitro and in vivo findings: 1) regulation of bone cell adhesion; 2) regulation of osteoclast function; and 3) regulation of matrix mineralization (Giachelli & Steitz, 2000).

### **Osteopontin and Cell Survival**

Osteopontin is a cell survival factor. Both soluble OPN, which works as a cytokine, and immobilized OPN, which function as an extracellular matrix protein, protect against apoptosis and induce survival and proliferation in several cell types (Standal et al., 2004). Several in vitro

studies have reported that OPN has a pro-survival and/or proliferative function in adherent cell types such as smooth muscle cells (Weintraub et al., 2000) and epithelial cells (Elgavish et al., 1998). Liu et al. (2009) reported that the overexpression of OPN in human embryo kidney-293 cells significantly increased the level of cell proliferation in vitro. Furthermore, despite diminished macrophage infiltration and interstitial fibrosis, the obstructed kidneys of OPN knockout mice exhibit increased levels of tubular cell apoptosis compared to wild-type mice, suggesting that OPN is capable of providing survival signals to tubular epithelial cells in vivo (Ophascharoensuk et al., 1999). The binding of OPN to the  $\alpha_v\beta_3$  integrin of endothelial cells activates the pro-survival transcription factor NF $\kappa$ B and protects endothelial cells from undergoing apoptosis (Scatena et al., 1998).

### **Osteopontin and Tumorigenesis**

Osteopontin is strongly associated with tumorigenesis (Standal et al., 2004). In patients with breast cancer (Singhal et al., 2004), multiple myeloma and prostate cancer (Saeki et al., 2003), high plasma levels or tumor expression of OPN are associated with poor prognosis. OPN is expressed by several types of cancer cells (Standal et al., 2004; Ue et al., 1998). Some of them also respond to OPN with enhanced survival and proliferation. A study indicated that epidermal growth factor (EGF) dependent proliferation of prostate cancer cells is amplified by OPN (Angelucci et al., 2004). This is probably due to an interaction of OPN with beta1 integrins on the surface of the cancer cells, leading to sustained activation of the epidermal growth factor receptor (EGFR) (Standal et al., 2004). Epidermal growth factor (EGF) is an important growth factor for prostate cancer cells and this interaction thereby promotes proliferation of the cancer cells. Similarly, IL-6 is an important growth promoting/survival factor for myeloma cells, and

OPN has been shown to promote myeloma cell growth in combination with IL-6 (Standal et al., 2004). Receptors involved are  $\alpha_4\beta_1$  and  $\alpha_v\beta_3$  (Abe et al., 2004) but the precise molecular mechanisms are currently not known. Although cancer cells often produce OPN, they also give rise to enhanced OPN production by host cells such as stromal cells/osteoblasts (Hullinger et al., 2000; Standal et al., 2004) and osteoclasts (Abe et al., 2004). OPN effects on tumor cells can thus be both autocrine and paracrine. Autocrine effects can influence cell proliferation and survival, converting benign tumor cells into highly metastatic cells (Oates et al., 1996), whereas paracrine effects can provide protection from cytotoxic macrophages, possibly by inhibiting the production of nitric oxide (Denhardt and Chambers, 1994). Collectively, these mechanisms could explain how tumor cells not expressing OPN are eliminated, while providing a mechanism of tumor cell escape and selection of OPN-producing clones in secondary metastases (Crawford et al., 1998). In this regard, higher median levels of plasma OPN in metastatic breast cancer have been associated with increased tumor burden and decreased survival (Singhal et al., 1997).

### **Osteopontin and Infant Nutrition**

Milk is an excellent source of nutrient (energy, macronutrients, vitamins and minerals) as well as bioactive proteins that play important roles in infant immunity. During the first phase of life, when infants are immunologically immature, breastfeeding provides an effective protection against infections (Lawrence & Pane, 2007); breast-fed infants have a reduced frequency of infectious episodes compared with formula-fed infants (Huffman & Combest, 1990; Dewey et al., 1995). This protective effect of human milk is likely achieved through the numerous bioactive components contained in the milk, which include proteins and peptides with antimicrobial and immune-stimulating properties such as lactoferrin, lactoperoxidase, lysozyme, and IgA (Hanson

et al., 2003; Field, 2005). However, a significant protein compositional dissimilarity exists between human milk and that of bovine milk and infant formulas (Hambreus et al., 1977). A less well-characterized bioactive protein that could potentially play a role in neonate and infant immunity and development is OPN.

As noted earlier, OPN is present in most tissues and body fluids, with the highest concentrations being found in human milk. Schack and his colleagues (2009) measured and compared OPN concentration in human milk, bovine milk, and infant formulas by enzyme-linked immunosorbent assay (ELISA). The OPN concentration in human milk was approximately 138 mg/L, which was considerably higher than the corresponding OPN concentrations in bovine milk (~18 mg/L) and infant formulas (~9 mg/L). The OPN levels in plasma from 3-months-old infants and umbilical cords were found to be 7 to 10 times higher than in adults (Schack et al. 2009). Moreover, isolated bovine milk OPN was shown to induce the expression of the Th1 cytokine IL-12 in cultured human lamina propria mononuclear cells isolated from intestinal biopsies (which represent the first line of the immune system at the gut mucosal surface) (Schack et al. 2009). Thus, the intestinal immune system may be modulated by ingested OPN. This is further supported by the observation that bovine and human milk OPN was resistant to proteolysis by neonatal gastric juice at pH 4.0 and above (Chatterton et al., 2004). Mean gastric pH has been reported to rise shortly after feeding in infants fed human milk, reaching pH values of 6.4 within 30 min after a bolus of human milk, gastric pH subsequently decreasing with time (Mason, 1962). Thus, part of the ingested OPN survives the passage through the infant's stomach and reaches the immune cells present in the intestine. The ingested OPN might further act directly against pathogenic bacteria in the gastrointestinal tract; OPN was able to opsonize bacteria, which leads to an enhanced phagocytosis (Schack et al., 2009). In a

recent study using preterm piglets, OPN enrichment decreased necrotising enterocolitis (NEC) severity relative to control formula, without any significant effects on intestinal morphology and digestive enzyme activities (Møller et al., 2011). Supplementation of infant formula with OPN could therefore potentially provide the infant with an important immunological factor.

## **1.2 Rotavirus and Rotavirus Vaccine**

### **Epidemiology of Rotavirus Gastroenteritis**

Among children in both the developed and developing world, rotavirus (RV) is the most commonly identifiable agent (Kapikian, 1993). In both the developed and developing world, it accounts for 25-50% of gastroenteritis requiring hospitalization in pediatrics (Bresee, 2008). Rotavirus gastroenteritis occurs almost exclusively in infants and children, with nearly every child having been infected by the age of 5 years (Bernstein, 2009). The majority of serious infections occur between 4 and 24 months of age (Podewils et al., 2004). Older children are protected from serious disease by previous exposure and apparent infection. Similarly, disease can occur in neonates, but is typically mild or asymptomatic due to protection from maternal antibodies (Glass et al., 2009).

### **Biology of Rotavirus**

Rotaviruses are non-enveloped viruses of the genus Reoviridae (Martella et al., 2010). The virus is characterized by a double-stranded RNA genome composed of 11 segments, which encode for six viral structural (VP) and six nonstructural proteins (NSP) (Greenberg et al., 2009; Martella et al., 2010). Rotavirus particles are icosahedrons, 70–75 nm in diameter, composed of

three concentric layers of structural proteins: the core, an inner capsid, and an outer capsid (**Figure 1.5**, Jayaram et al., 2004). The outer capsid is composed of a viral protein 7 (VP7) coating with VP4 spikes protruding from the viral surface (Greenberg et al., 2009). This layer is the most antigenically important portion of the virus, with the VP7 glycoprotein (G-type antigen) and VP4 protease-sensitive protein (P-type antigen) being the major immunological targets of the human immune system (Greenberg et al., 2009). The inner capsid is composed solely of VP6. Based on the antigenic properties of VP6, rotaviruses are broken into 7 serogroups, denoted A–G. Serogroup A is the only serogroup that commonly causes human disease (Santos & Hoshino 2005; Martella et al., 2010). Conventionally, rotavirus strains are denoted by the P serotype name first, followed by its genotype in brackets, followed by the G-type (Clark et al., 2008). Common serotypes of RV infected humans include P[8]G1, P[4]G2, P[8]G3 and P[8]G4 (Gentsch et al., 1996).

### **Transmission and Pathogeneses of Rotavirus**

Rotavirus is believed to be transmitted via the fecal oral route, close personal contact, and contact with contaminated environmental sources. However, the prevalence of RV in high-income countries despite improved sanitation suggests that nonfecal routes play a role in transmission, such as respiratory droplets (de Wit et al., 2003; Santosham et al., 1983)

Upon ingestion, the RV targets the epithelial lining of the intestine (Greenberg et al., 2009). During viral replication, the segments of the viral genome are disassociated from viral proteins and one another; this allows reassortment of genotypes in cells infected with more than one strain of RV (Desselberger et al., 2009). Rotavirus disrupts the normal functioning of the gastrointestinal mucosa through a number of mechanisms (Greenberg et al., 2009); the most

potent is the viral enterotoxin non-structural protein 4 (NSP4), which alters the permeability of the gut mucosa by weakening the tight junctions between cells, disrupting the cytoskeleton of the infected cells, increasing the secretion of chlorine ions, and stimulating the gut motility through the enteric nervous system (Ramig, 2004), which causes in an uncontrolled outflux of water into the intestinal lumen, resulting in profuse diarrhea.

### **Clinical Symptoms**

Rotavirus disease is most commonly characterized by acute gastroenteritis. After an incubation period of 1-3 days (Bernstein, 2009), RV infections present with symptoms common to many enteric pathogens: profuse watery diarrhea, vomiting, and fever (Staat et al., 2002). The severity of symptoms varies, but on average is more severe than other viral enteric pathogens (Intusoma et al., 2008). It accounts for 25%–40% of diarrheal cases requiring hospitalization (Podewils et al., 2004). Diarrhea typically lasts from 4 to 7 days (Desselberger et al., 2009). Virus-specific diagnostic tests and treatment are not necessary as symptoms typically resolve within a week of disease onset (Cheng et al., 2005). However, supportive care, primarily rapid rehydration, is critical to preventing complications. The most common complication associated with RV infection is dehydration. Severe dehydration is characterized by reduced or altered consciousness, lack of urine output, low blood pressure, weak quickened pulse, cool moist extremities, and peripheral cyanosis (Duggan et al., 1992). Malnutrition and diarrhea form a destructive cycle in children and as the most common diarrheal pathogen in infants, RV plays an important role in this cycle. Nutritional support is a critical component of ameliorating the short-term and long-term consequences of RV, as well as other diarrhea diseases.

## Treatment of Rotavirus Infection

Dehydration is responsible for 90% of deaths from infectious diarrhea (Claeson et al., 1990). Treatment for RV gastroenteritis, as with the majority of infectious watery diarrhea, is supportive; children should receive appropriate hydration and nutritional support. The current World Health Organization (WHO) recommendation is for low-osmolarity oral rehydration solution (ORS, 245 mOsm/L) with 75 mmol/L each of sodium and glucose. In cases of severe dehydration in which the child is unable to drink, intravenous fluids should be used for initial management, with the institution of ORS as soon as possible. The WHO also incorporated early refeeding into their recommendations for diarrhea treatment (Chandran et al, 2010).

Antimicrobial therapy should be used only in specific cases of infectious diarrhea and in general do not have a role in the treatment of RV gastroenteritis. Probiotics, microorganisms believed to restore microbial balance in the gastrointestinal tract, may have a role in the treatment of diarrhea by enhancing the immune response or providing a competitive blockage of pathogen receptor sites. In a meta-analysis of seven clinical trials, *Lactobacillus* GG (LGG) was shown to reduce the duration of rotavirus diarrhea by 2.1 days (95% CI: 3.6-0.6) (Szajewska et al., 2007); however, a recent trial in rural India did not show any efficacy (Misra et al., 2009). *Saccharomyces boulardii* and *Lactobacillus reuteri* have also shown beneficial effects for RV gastroenteritis (Guandalini, 2008). Additional studies are needed to further define the necessary dose and duration of treatment, particularly in developing country settings. Although the morbidity and mortality from rotavirus gastroenteritis differ across socioeconomic strata, rates of illness are similar across developed and developing country settings. This indicates that hygiene and sanitation improvements, which have been credited with reducing incidence of most causes of infectious diarrhea in developed countries, are unlikely to prevent RV disease significantly



(Dennehy, 2008). Therefore, prevention in the form of vaccines is essential for the control of RV disease.

### **Rotavirus Vaccine**

Children vaccinated with oral live vaccines develop a humoral immune response and are protected against severe disease upon reinfection. Protection against rotavirus infection has been associated with the presence of antirotavirus IgA antibodies in the gastrointestinal mucosal surface (Fischer et al, 2002). Although levels of serum anti-RV IgA do not always correlate with levels of IgA antibody in the gut, the IgA response is often used to measure vaccine immune response. A few studies have demonstrated that both presence of serum IgA and presence of serum IgG are protective. O’Ryan et al. (1994) reported that children who were infected during the second season of the study had significantly lower geometric mean titers of RV IgA and IgG than did those who were not subsequently infected.

Considerable efforts have been made over the last 30 years to develop safe and effective rotavirus vaccines for human infants (El-Attar et al., 2009). In December 2009, WHO recommended routine immunization of infants for prevention of RV disease. Currently, two RV vaccines, Rotarix<sup>®</sup> (GlaxoSmithKline, Research Triangle Park, NC) and Rotateq<sup>®</sup> (Merck & Co Inc, Whitehouse Station, NJ), based on an attenuated G1P [8] human rotavirus or a mixture of five human-bovine reassortant rotaviruses of different G-types, have been developed and licensed for use in both developing and developed countries.

Rotarix is a monovalent vaccine composed of an attenuated human rotavirus strain G1P[8]. Three clinical trials involving 5,024 infants conducted in Finland, Latin America and Singapore indicated a good immunogenicity of this vaccine (De Vos et al., 2004). Many reports

indicate that serum IgA antibodies are important for immunity to rotavirus and resistance to symptomatic re-infection (Yuan et al., 1996; Johansen & Svensson, 1997). In the 3 trials, 61–91% of vaccinated infants developed RV-specific serum IgA antibodies after 2 doses, depending on Rotarix® potency (De Vos et al., 2004). Rotarix significantly decreased RV gastroenteritis episodes (over 85%~96% protection against moderate to severe gastroenteritis) and related hospitalizations in vaccinated infants, as compared to placebo (De Vos et al., 2004). In a recent trial conducted in healthy Indian infants, Rotarix increased RV-specific IgA concentrations more than two-fold in 60.5% of infants, while 7.5% of placebo recipients showed a two-fold increase (Narang et al., 2009). Thus, this vaccine proved to be nonreactogenic, well tolerated and not associated with intussusception. This oral rotavirus vaccine is administered in two doses at approximately 6-24 weeks of age (O'Ryan, 2007). The first dose may be administered from the age of 6 weeks and there should be an interval of at least 4 weeks between doses (O'Ryan, 2007). The vaccination course should preferably be given before 16 weeks of age and must be completed by the age of 24 weeks (O'Ryan, 2007).

### **1.3 Gastrointestinal Development and Piglet Model**

#### **General Overview**

The small intestine is the largest component of the digestive tract and the major site of digestion and absorption. It is divided into three parts, the duodenum, the jejunum and the ileum.

The epithelium of the small intestine consists of the following cell types:

Enterocytes or absorptive cells. The predominant cell type of the epithelium is the enterocyte or absorptive cell. They are tall columnar cells with microvilli and a basal nucleus,

specialized for the transport of substances. Amino acids and monosaccharides are absorbed by active transport, monoglycerides and fatty acids cross the microvilli membranes passively. Absorbed substances enter either the fenestrated capillaries in the lamina propria just below the epithelium, or the lymphatic lacteal (most lipids and lipoprotein particles). Enterocytes have a lifespan of about 5-6 days.

Goblet cells. These mucus-secreting cells are the second most abundant epithelial cell. They are found interspersed among the other cell types. Their mucous is a very large glycoprotein that accumulates at the apical end of the cell, rendering it wide. The slender base of the cell holds the nucleus and organelles. Goblet cells usually appear pale or empty due to the loss of their contents upon preparation. Their lifespan is also 5-6 days.

Paneth cells. Paneth cells are found only in the bases of the crypts. These cells have an oval basal nucleus and large, refractile acidophilic granules at their apical end. Paneth cells also phagocytize some bacteria and protozoa. They may have a role in regulating intestinal flora. They have a lifespan of about four weeks.

Enteroendocrine cells. They are most often found in the lower part of the crypts but can occur at all levels of the epithelium. Their most abundant products here are cholecystokinin or CCK, which stimulates pancreatic enzyme secretion.

M or microfold cells. These cells are epithelial cells that overlie Peyer's patches and other large lymphatic aggregations. They are relatively flat and their surface is thrown into folds, rather than microvilli. They endocytose antigens and transport them to the underlying lymphoid cells where immune responses to foreign antigens can be initiated.

Undifferentiated cells. These stem cells are found only at the base of the crypts and give rise to all the other cell types. A cell destined to be a goblet cell or enterocyte undergoes about 2

additional divisions after leaving the pool of stem cells, and migrates from the crypt to the villus. It will be shed at the tip of the villus.

### **Cell Proliferation**

Crypt cell proliferation is markedly increased in sow reared piglets in the first two days of life followed by decrease at day 7 and week 12 (Zabielski et al., 2008). The proliferative crypt cells are higher in DNA content while the villus epithelial cells have more protein (Fan et al., 2001). The typical life span of enterocytes in the proximal small intestine is twice as long as the distal in 14-to 18-day old piglets fed sow milk replacer formula (Fan et al., 2001). At birth, the gastrointestinal tract is immature in the piglet, but villus height is considerable in length (Thymann et al., 2006). Small intestinal morphology has previously been used as a marker of intestinal health in piglets by extensive studies (Chen et al., 2005; Houle et al., 1997; Oliver et al., 2002).

### **Digestive Enzyme Activity**

Sucrase-isomaltase (EC 3.2.1.10) and lactase-phlorizin hydrolase (EC 3.2.1.23-62) are digestive enzymes located on the brush border membrane facing the small intestinal lumen and are used as a marker of digestive capacity in the small intestine, as well as serving as indicators of enterocyte maturity (Adeola & King, 2006).

Lactase-phlorizin hydrolase (LPH) is the major intestinal disaccharidase responsible for the hydrolysis of the lactose in milk and is, thus, an important factor in energy utilization in developing mammals. The expression of LPH activity is developmentally programmed and reaches a maximum late in gestation, presumably to prepare the newborn for digestion of milk

lactose (Sangild et al., 2000; Buller et al., 1990). In the neonatal intestine, several factors have been shown to affect the expression and activity of LPH, including diet (Oliver et al., 2002), the proximal-distal location (Houle et al., 1997) and stage of enterocyte differentiation along the crypt-villus axis (Rings et al., 1992). At the cellular level, the production of LPH is subject to multiple sites of regulation at the transcriptional, translational and posttranslational levels (Burrin et al., 2001). In pigs, at least two isoforms of prolactase-phlorizin hydrolase (pro-LPH), one mannosylated and the other bearing complex glycosidic side chains, have been isolated from the small intestinal mucosa. Furthermore, the complex glycosylated precursor form is cleaved proteolytically before insertion into the brush-border (BB) membrane as BB-LPH (Burrin et al., 2001).

Lactase and sucrase have been reported to increase significantly before birth in the last 20 % gestation (Sangild et al., 2002). Lactase activity in a newborn piglet is high at birth followed by a decline within the first 2 months of age (James et al., 1987). This decline may be delayed if the post weaning diet contains high amounts of lactose (Marion et al., 2005). Sucrase, which is a part of the sucrase-isomaltase complex, is nearly absent at birth until approximately 4-6 days of age, followed by increases in activity with age (Adeola & King, 2006; James et al., 1987).

In summary, morphology and digestive enzyme activity serve as good markers of structural and functional capacity in the small intestine. At birth, the GI is relatively immature, but immediate introduction of nutrients promotes villus growth, protein synthesis, cell proliferation, as well as maturation and development in the activity and expression of brush border enzymes. As cells proliferate and migrate along the crypt-villus axis, they differentiate to mature enterocytes with increased expression of enzymes. Diet is associated with the regulation

of these digestive enzymes through transcription, translation, and posttranslational events, suggesting the potential role in modulating brush border enzyme activities.

### **Piglet as a Model for the Infant Intestinal Development**

The piglet model is a long established model used for both enteral and parenteral nutritional studies of human neonates (Moughan et al., 1992; Wykes et al., 1993). Compared with rodents, the neonatal pig has more anatomical, physiological, immunological, and metabolic similarities with the human infants (Moughan, et al., 1992).

In the piglet model, the small intestinal weight increases by 70-80 % in the last 3-4 weeks of gestation, coinciding with increase of tissue specific activities of brush border enzymes (Bjornvad et al., 2005). The small intestine undergoes tremendous growth and functional changes in the immediate pre- and postnatal periods and these changes may be diet-dependent (Bjornvad et al., 2005). In preterm piglets, formula-feeding is associated with decreased intestinal growth and brush-border enzyme activities, compared to colostrum-feeding (Bjornvad et al., 2005).

### **Piglet as a Model for RV vaccination**

Due to the impervious nature of the sow placenta to immunoglobulins pigs are born devoid of maternal antibodies (MatAb), acquiring serum Igs by intestinal absorption of colostrum Igs for up to 36 h after birth before gut closure (cessation of absorption of intact Ig) occurs (Banks & McGuire, 1989), facilitating experimental manipulation of levels of maternal antibodies.

## **Interference of Neonatal Active Immune Responses by MatAb**

Vaccination of neonates faces many challenges due to the immaturity of the neonatal immune system and interference by maternal antibodies (MatAb) present at vaccination. Interference of vaccine-induced immune responses by MatAb has been reported for both live vaccines and non-replicating vaccines. The degree of interference may depend on the ratio between the amount of MatAb and the antigen present (Arias et al., 2001). Optimal amounts of MatAb can enhance B-cell responses by forming antigen-antibody complexes that induce complement deposition. On the other hand, neutralizing MatAb neutralize live vaccines and reduce the antigen mass available for immune response induction. Thus, the outcomes of MatAb effects, whether positive (enhancing) or negative (suppressing) are dependent on the MatAb titers, the vaccine, and the dose and type of antigen (Arakawa et al., 1998). Rotavirus infections occur as early as under 3-months of age in infants even when the levels of MatAb in the circulation (acquired from placental transfer) and intestine (acquired from breast milk) remain high.

The variable efficacies and seroconversion rates in many rotavirus vaccine trials in human infants were influenced by the effects of MatAb on vaccine take and immunogenicity (Andrew et al., 1992). Various titers of RV specific MatAb at vaccination have been shown to influence the outcome of active immunity. Nguyen et al. (2006) reported that high titers of circulating maternal antibodies suppressed effector and memory B-cell responses induced by an attenuated (Att) human RV (HRV) priming and RV-like particle-immunostimulating complex (VLP-ISCOM) boosting vaccine regimen. Virus-like particles (VLP) offer attractive approaches for neonatal RV vaccines due to their potential safety (non-replicating so lack of infectivity) compared to live vaccines. However, low titer maternal antibodies (LoMatAb) had both

enhancing and suppressing effects on B cell responses to a combined live attenuated HRV and VLP-ISCOM vaccine, depending on tissue, antibody isotype and vaccine (Nguyen et al. 2006). In Nguyen's study on the AttHRV/VLP (replicating) vaccine, LoMatAb enhanced intestinal IgM and IgA antibody secreting cell (ASC) numbers at various times (pre- and post- challenge). However, there was evidence for suppression of systemic antibody responses by LoMatAb, especially systemic IgA ASC, IgA memory B cells in peripheral blood and serum IgA antibody responses. Therefore, the AttHRV/VLP vaccine partially overcame LoMatAb suppression, conferred moderate protection against virulent HRV and represents a new candidate for rotavirus vaccines for both humans and animals (Nguyen et al. 2006).

### **Piglet Models for RV Infection and Vaccination**

Pigs are the only animals in which diarrhea can be induced by HRV strains, which make them more relevant for the study of RV vaccines for use in human infants. The window of susceptibility of pigs is longer (up to 8 weeks or more after birth), which allows studies of the immune responses and the evaluation of vaccine efficacy with booster doses (Richards et al., 1998; Ward et al., 1992). Conventional pigs (raised under normal conditions) have been used by many investigators, but pigs varied in age, the presence or absence of MatAb (colostrum fed or deprived pigs) and the confounding effects of extraneous RV infectious. Therefore, there are currently two pig models for the study of RV induced protection and immune responses: the gnotobiotic pigs (raised in germ-free conditions) and colostrum-deprived, cesarean-derived pigs.

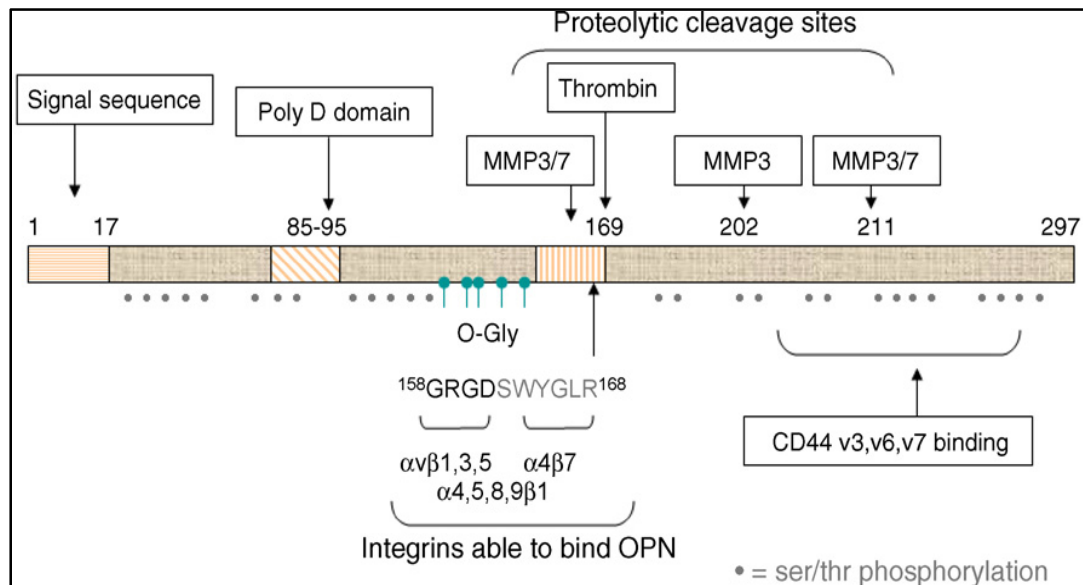
Gnotobiotic Piglet Model. Neonatal Gnotobiotic (Gn) pigs have been used extensively to study immune responses to RV, RV vaccines and the correlates of protective immunity. Studies were conducted of the magnitude and kinetics of IgM, IgA and IgG ASC in pigs inoculated with



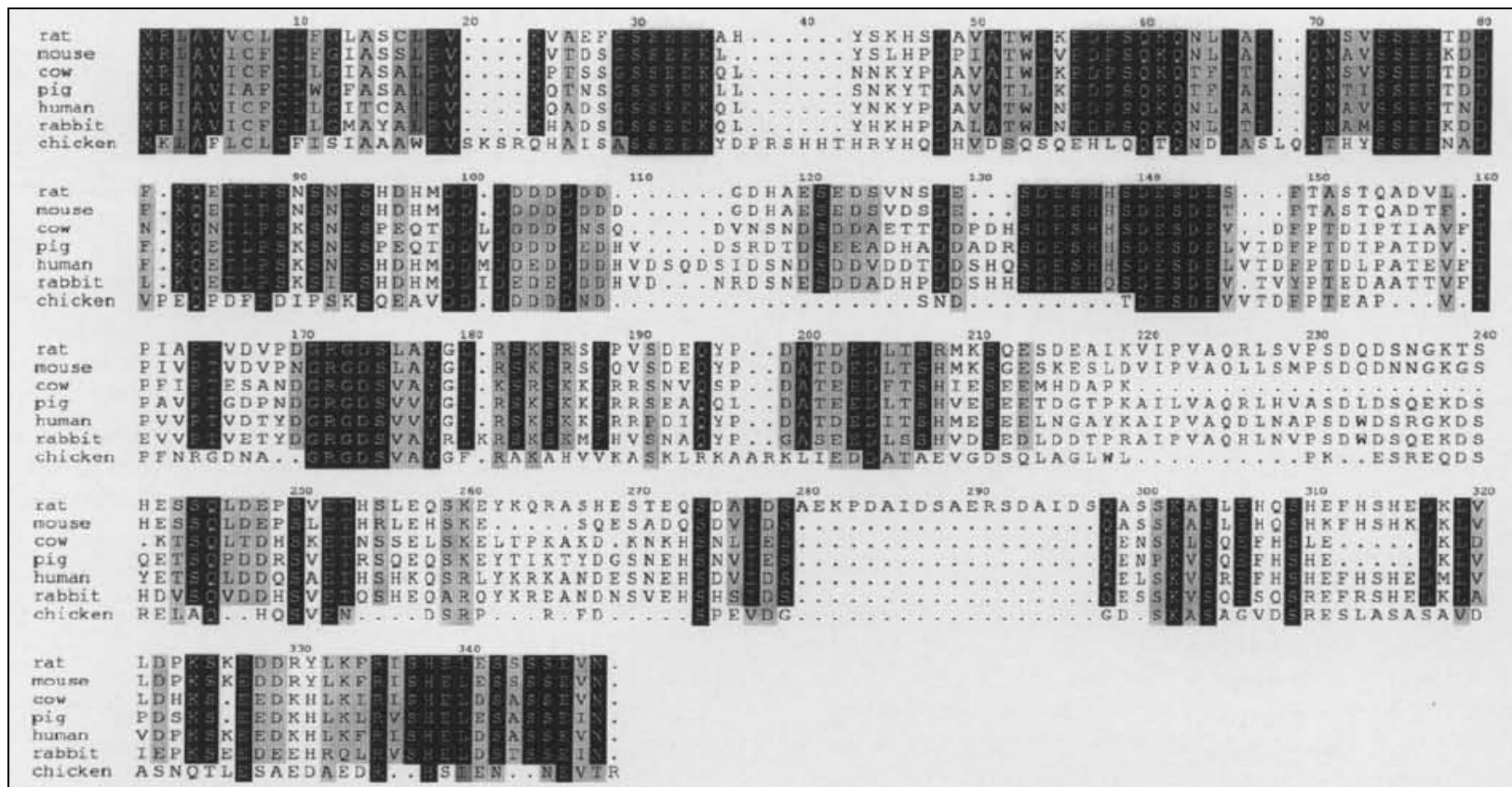
porcine RV SB1A and Gottfried strains or HRV Wa strain using an ELISPOT assay (Chen et al., 1995). The IgM ASC appears as early as 3 days after infection and peak at 7 days post-inoculation (PID) in MLN and spleen and later in lamina propria (LP) of the intestine. The IgA and IgG ASC numbers peak later at PID 14-21 and the IgA ASC responses are found mostly in intestinal lymphoid tissues. The magnitude of the antibody responses is highest in the tissues close to the site of viral replication, i.e. the intestine. In the Gn pig model, a correlation of protection against infection and disease has been associated with intestinal IgA ASC, whereas the IgA ASC and antibodies in blood can serve as indicator of the IgA intestinal response, and hence protection (Yuan et al., 1996). The route of vaccine delivery has also been investigated intensively in the Gn model. The administration via mucosal routes has shown clear advantages over the systemic route in inducing mucosal antibody responses as well as in the ease of delivery of the vaccine. The order of the routes used for priming and boosting also determines the level of protection in pigs. Oral priming and intranasal boosting strategies used in rotavirus vaccine studies of gnotobiotic piglets were highly effective in inducing antibody responses, presumably due to exploiting multiple mucosal inductive sites. This strategy of avoiding mucosal sites where booster vaccines may be neutralized by local antibodies induced by the priming vaccine assures that booster vaccination will evoke effective antigenic stimulation (Yuan et al., 2001). Although used as a well-established model, Gn pigs require expensive surgical procedures and the specific pathogen-free facilities are not always available.

Colostrum-Deprived, Cesarean-Deprived Piglet Model. An alternative to Gn pig model is the use of colostrum-deprived, artificially-reared piglets, which possess similar susceptibility to RV infection, clinical signs and intestinal pathology as human infants or Gn pigs (Gomez, 1997; Zijlstra, 1999). An advantage of this model over the Gn piglet is bacterial colonization of

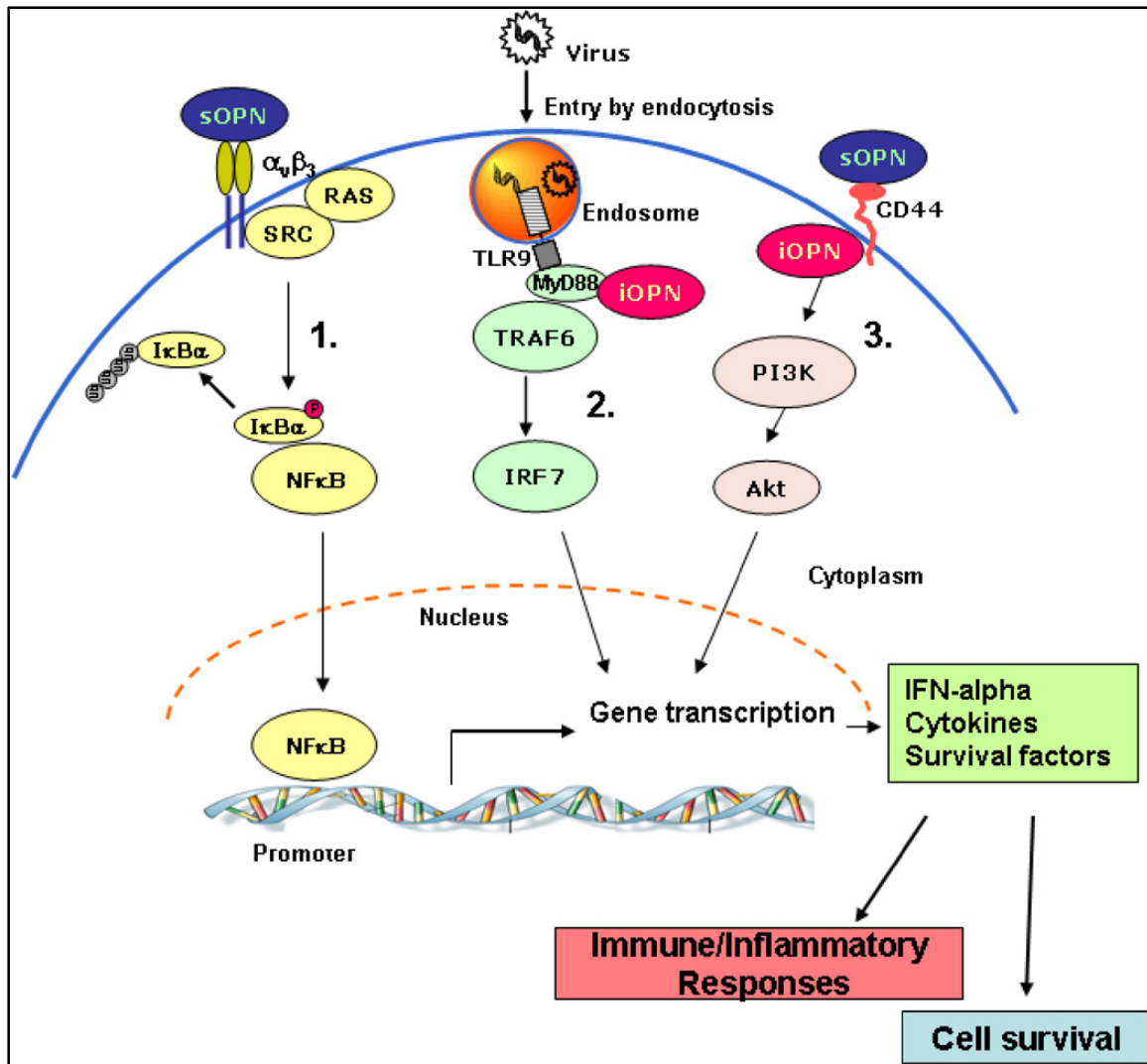
the intestinal tract, which is critical to the intestinal barrier development and protection of the host against pathogens (Chen & Walker, 2005). Moreover, in order to investigate potential nutritional interventions, the presence of the intestinal microbiota, human infants would exhibit, is critical. One of the limitations of this model is its susceptibility to infection by a wide variety of pathogens including RV. Bovine colostrum has a critical role in postnatal health as an immune booster. Bovine colostrum is an extremely important source of immunoglobulins; the concentrations of IgG, IgM, and IgA are 100-fold higher than in normal milk (Rooke and Bland, 2002). Bovine colostrum supplementation has been shown to increase total serum IgA concentrations in weaned piglets (Boudry et al., 2007). Therefore, bovine colostrum was used in our first oral OPN supplementation study.



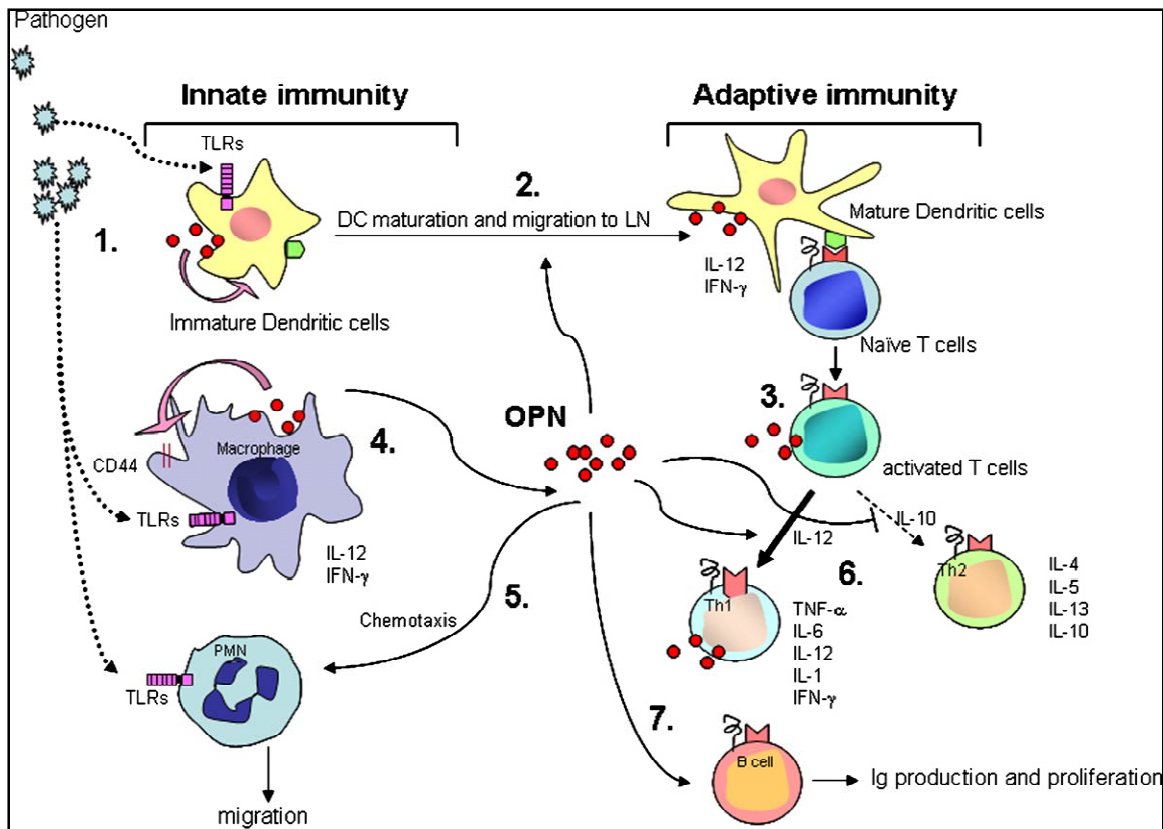
**Figure 1.1 OPN structure** (Wang & Denhardt, 2008). From left to right are indicated: the N-terminal signal sequence regulating OPN secretion that is cleaved off when the protein is secreted; a region rich in aspartate residues responsible for strong binding to hydroxyapatite; the sites of O-glycosylation N-terminal to the GRGD sequence responsible for integrin interactions; the site of cleavage by thrombin immediately C-terminal to the YGL sequence; the likely region of interaction with CD44 variants towards the C-terminal portion of the protein. Sites susceptible to cleavage by matrix metalloproteinases are also noted; grey dots indicate potential regions of serine/threonine phosphorylation scattered throughout the protein.



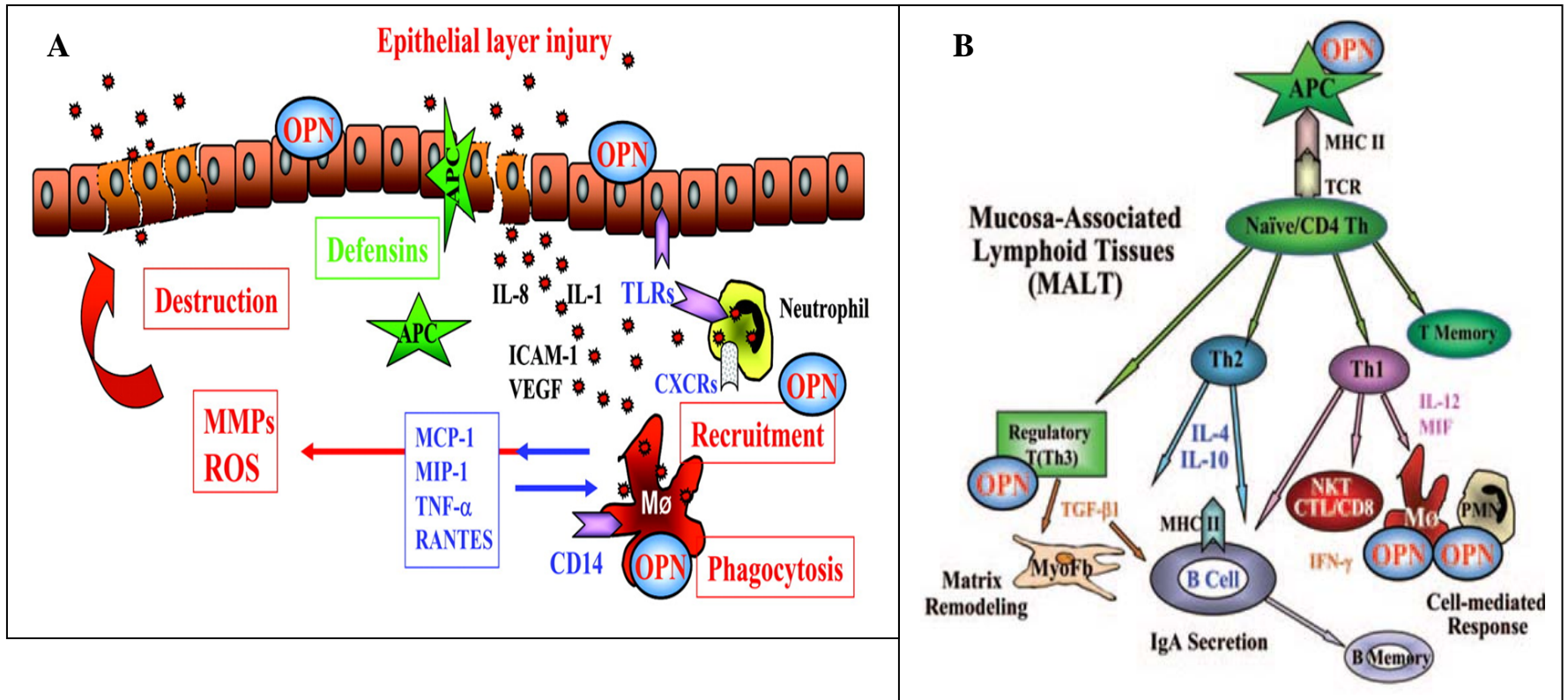
**Figure 1.2 Comparison of osteopontin protein sequences.** The amino acid sequences from six mammalian (rat, mouse, cow, pig, human, and rabbit) and one avian (chicken) species have been aligned by means of the multiple alignment program of Thompson et al. (1994) to maximize identical (dark shading) and similar (light shading) amino acids identified by JavaShade (Southern and Lewis, 1998; <http://industry.ebi.ac.uk/JavaShade/>) to reveal conserved structures. The 16-amino-acid leader sequence beginning with a methionine (position 1) is included with the first amino acid of the processed protein starting at position 17.



**Figure 1.3 OPN interacts with different signaling pathways to mediate immune responses** (Wang & Denhardt, 2008). (1) Engagement of extracellular OPN (sOPN in blue) to integrin  $\alpha_v\beta_3$  signals through the Src and FAK tyrosine kinases to activate transcription factor NF $\kappa$ B. OPN promotes phosphorylation of I $\kappa$ B $\alpha$ , which leads to the degradation of I $\kappa$ B $\alpha$  and nuclear translocation of NF $\kappa$ B which regulates the transcription of a variety of pro-inflammatory cytokines and mediators (Vejda et al., 2005). (2) In plasmacytoid DCs, intracellular OPN (iOPN in red) is associated with MyD88 during TLR9 engagement with viral DNA in the endosome, promoting TLR9 signaling toward IRF7 rather than IRF-5/NF $\kappa$ B, which would allow for robust IFN- $\gamma$  production (Shinohara et al., 2006). (3) OPN interaction with CD44 activates PI3k/Akt signaling, which can mediate cell survival (Packer et al., 2006).



**Figure 1.4 OPN's role in the regulation of innate and adaptive immunity** (Wang & Denhardt, 2008). (1) Exogenous pathogens activate TLRs on the surface of cells, including macrophages, neutrophils and immature dendritic cells. OPN is secreted by macrophages and dendritic cells when challenged by foreign antigens and enhances the activation and functions of these cells. OPN promotes neutrophil migration towards the site of injury. (2) OPN promotes immature dendritic cells to mature and migrate to draining lymph nodes, where they present processed antigens through the MHC to naïve T cells and initiate a cell-mediated immune response. (3) Signals from DCs activate naïve T cells and determine the polarization of T cells to Th1 or Th2 type cytokine responses. (4) Macrophages produce large amounts of OPN, which in an autocrine/paracrine manner contributes to the migration of macrophages and the expression of the pro-inflammatory cytokine IL-12. (5) OPN produced by various immune cells at inflammatory sites promotes infiltration of neutrophils. (6) Activated T cells are promoted by IL-12 to differentiate towards the Th1 type, producing Th1 cytokines (IL-12, IFN-γ). OPN inhibits production of the Th2 cytokine IL-10, which leads to an enhanced Th1 response. (7) OPN promotes B lymphocyte proliferation and immunoglobulin production.

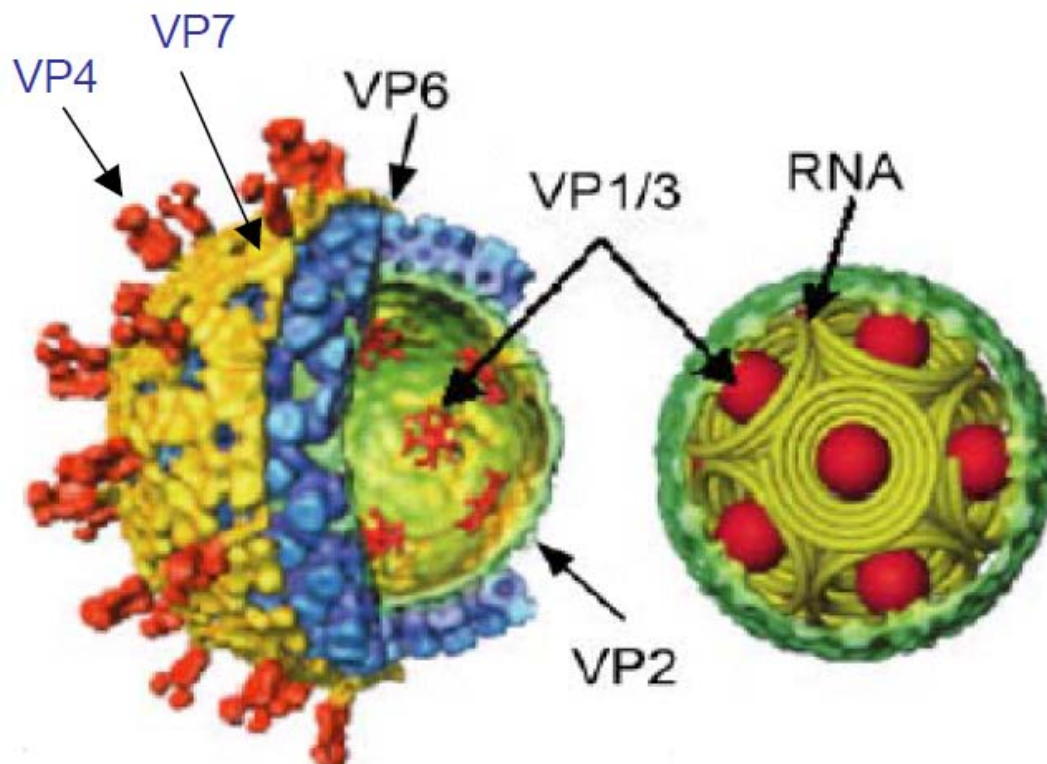


**Figure 1.5 Possible effects of OPN on the innate and adaptive immune responses in mucosal tissues** (Sodek et al., 2006).

A. OPN expressed by epithelial and immune cells acts as a chemoattractant to macrophages and neutrophils and regulates their phagocytic activity, reactive oxidative burst (ROS) and release of cytokines, and proteolytic enzymes.

B. OPN can modulate the inflammatory reaction and promote repair through its effects on T-cell differentiation and by its ability to influence the survival of epithelial cells, macrophages, and fibroblasts.





**Figure 1.6 Rotavirus structures.** Rotavirus particles are icosahedrons, 70–75 nm in diameter, composed of three concentric layers of structural proteins: the core, an inner capsid, and an outer capsid. The outer capsid is composed of a viral protein 7 (VP7) coating with VP4 spikes protruding from the viral surface (Greenberg et al., 2009). This layer is the most antigenically important portion of the virus, with the VP7 glycoprotein (G-type antigen) and VP4 protease-sensitive protein (P-type antigen) being the major immunological targets of the human immune system (Greenberg et al., 2009). The inner capsid is composed solely of VP6. Based on the antigenic properties of VP6, rotaviruses are broken into 7 serogroups, denoted A–G (Jayaram et al., 2004).



## Chapter 2

### Effect of Osteopontin on Intestinal Development of Neonatal Piglets

#### Abstract

The objectives of this study were to investigate the effects of osteopontin (OPN) on piglet intestinal development. Newborn, colostrum-deprived piglets (n=27) were randomized to receive three treatments: formula with bovine OPN (**OPN**; 140 mg/L); formula alone (**FF**); or sow reared (**SR**) for 21 days. Body weight, intestinal weight and length, mucosal protein and DNA content, disaccharidase activity, villus morphology, and crypt cell proliferation were measured. Statistical significance was assigned at  $P < 0.05$ . No significant effects of OPN were observed for body weight, intestinal weight and length. Mucosal protein content of SR piglets was lower than FF and OPN piglets in the duodenum, but higher than FF and OPN piglets in the ileum. No significant effects of diet in mucosal DNA content were detected for the three regions of the small intestine. Lactase and sucrase activities of SR piglets were higher than the two formula-fed groups in the duodenum, lower in the ileum. No significant differences in lactase and sucrase activities were noted between FF and OPN piglets in the duodenum and ileum. FF piglets had higher jejunal lactase activity than SR piglets, whereas no significant effect of diet was observed in jejunal sucrase activity among the three groups. Duodenal and ileal villus length and villus area of SR piglets were lower than two formula-fed groups, while OPN piglets did not differ from FF piglets. There was a significant effect of diet ( $P < 0.0001$ ) on jejunal crypt cell proliferation, with proliferation in OPN piglets being intermediate between FF and SR. In summary, supplemental OPN increased jejunal crypt cell proliferation, independent of evident

morphological growth, and had a minor impact on lactase activity in the small intestine of neonatal piglets.

## **Introduction**

Human milk is an excellent source of nutrition for infants and it contains a wide variety of bioactive proteins that are known to stimulate cellular growth and differentiation *in vivo* and *in vitro* (Grosvenor et al., 1993; Donovan et al., 1994). However, a significant difference in protein composition exists between human milk, bovine milk and infant formulas (Hambreis et al., 1977). Human breast milk is the optimal form of nutrition for human infants (AAP, 1995); hence, efforts have been made to improve the composition of infant formulas to more closely mimic that of breast milk. Purifying proteins from bovine milk and supplementing these to infant formula has been done, for instance, adding  $\alpha$ -lactalbumin (Trabulsi et al., 2011). However, a less well-characterized bioactive protein in milk that could potentially play a role in gastrointestinal growth and development in infants is osteopontin (OPN).

Osteopontin is a multifunctional protein that is implicated in a wide number of biological processes including cell survival, bone remodeling, and immune modulatory functions (Sodek et al., 2000). The protein is widely expressed in variety of tissues and is present in body fluids including blood, bile and milk (Sodek et al., 2000). It is expressed by a single gene in a cluster of SIBLING family proteins that share structural and functional properties (Fisher and Fedarko, 2003) on the long arm of chromosome 4 in humans and chromosome 5 in mice. Osteopontin is synthesized as a ~ 34-kDa protein that is extensively modified by phosphorylation, glycosylation and sulfation prior to its secretion as a protein that can range in size from 44- to 75-kDa (Sodek

et al., 2000). The heterogeneity of the secreted protein reflects differences in these post-translational modifications, which have been related to different functional activities associated with mineralization (Sodek et al., 2000) and cell attachment and signaling (Ashkar et al., 2000). However, most of the known functional activities of OPN can be attributed to highly conserved structural motifs (RGD sequence) involved in cell-surface CD44 and integrin receptors. Interactions between the centrally located RGD sequence (arginine-glycine-aspartate) and the  $\alpha_v\beta_3$  integrin, which is highly expressed in macrophages and osteoclasts, have been well-documented (Sodek et al., 2006). Binding through integrin receptors, OPN modulates a variety of cellular activities, including cell migration and proliferation, survival, motility and phagocytosis, all of which involve the cytoskeleton and have an impact on the inflammatory responses of macrophages and the fibrotic activities of fibroblasts (Sodek et al., 2006). Schack et al. (2009) demonstrated that the OPN concentration in human milk is in a range of  $138 \pm 79$  mg/L, which is considerably higher than OPN concentration in bovine milk and infant formulas. Although OPN is present at a relatively high concentration in human milk, little research has been conducted to assess its potential role in gastrointestinal growth and development of infants. Previous studies from our laboratory demonstrated that the addition of OPN to infant formula modulated intestinal gene expression in neonatal Rhesus monkeys (Donovan et al., 2011). There was a remarkable difference in jejunal gene expression between monkeys fed standard formula vs. mother's milk (1025 genes), whereas OPN supplementation reduced the difference in gene expression relative to BF by over 5-fold from 1025 to 225 genes. However, the intestinal tissue samples were not collected in this study and, thus, the changes in intestinal structure and function were unknown.

Our objective was to determine whether supplemental OPN have an impact on gastrointestinal structure and functional development in the piglet, a commonly used preclinical model for infant nutrition (Ebert et al., 2005). The human infant and neonatal piglet are similar in anatomy, physiology and disaccharidase levels (Chen et al., 2005; Walsh et al., 2009). We hypothesized that supplementation of OPN to formula will influence intestinal development of piglets to be more similar to sow-reared piglets, compared to piglets fed with formula alone.

## **Materials and Methods**

### *Chemicals*

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise indicated.

### *Study Design and Dietary Treatments*

All animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Illinois. Newborn, colostrum-deprived piglets (n=27) were obtained by Cesarean section of term gestation sows (n=3), weighed, and littermates were randomized into one of three dietary groups: formula alone (FF, n=9), formula with osteopontin (OPN, n=8), or sow-reared (SR, n=10). Formula-fed piglets were administered bovine colostrum (Colostrum Plus, Jorgensen Laboratories, Inc., Loveland, CO) in five feedings of 30 ml/feeding for the first 24 hrs. Powdered bovine colostrum was rehydrated at 350g/1.4L formula. Piglets were then fed a nutritionally-complete commercial piglet formula (Advance Liquiwean, MSC, Dundee, IL) comprised of 25% protein, 13% fat, 44% lactose, 6.5% ash, and containing levels of vitamins,

minerals, and trace elements to meet NRC requirements for piglets' growth (NRC, 1998). On d2 postpartum, piglets were administered 1ml of iron dextran (Durvet, Inc Blue Springs, MO) by intramuscular injection. Diets will be prepared as 20% solids with the addition of double distilled water in a blender (200g/Liter). Therefore, protein, fat and lactose were about 5%, 2.6% and 8.8% in the final formula, respectively. The bovine OPN was provided as a dried, purified powder by Arla Foods Ingr. (Vibjy P/S, Denmark). The powder is ~ 83% purified OPN by weight. It was diluted in double distilled water and was added to the formula immediately before feeding at a final concentration of 116 mg/L. Formula was offered at 360ml/kg/day divided into 14 equal feedings. Formula intake, stool consistency and activity scores were assessed daily (Correa-Matos et al., 2003). Formula not consumed by the piglet was recorded at each feeding in order to accurately determine the volume of formula and OPN consumed daily.

#### *Sample collection*

On d21 postpartum, piglets were injected intraperitoneally with 5-ethynyl-2'-deoxyuridine (EdU; Invitrogen Molecular Probes, Eugene, OR) at a dose of 2.5 mg/kg body weight at 2 hrs prior to euthanasia to measure rates of enterocyte proliferation. 5-ethynyl-2'-deoxyuridine is a nucleoside analog of thymidine and is incorporated into DNA during active DNA synthesis. Piglets were sedated with Telazol (Tiletamine HCl and Zolazepam HCl, 3.5mg/kg BW each, Pfizer Animal Health, Fort Dodge, IA) and then euthanized by an intracardiac injection of sodium pentobarbital (Fatal Plus, Vortech Pharmaceuticals, Dearborn, MI; 72mg/kg body weight). Blood and tissue samples were collected for analysis. Blood was collected by cardiac puncture into heparin-laced vials and serum was separated by centrifugation. A laparotomy was performed and the small intestine was quickly excised from the pyloric

sphincter to the ileocecal valve and its length was measured. The intestine was cut into three segments at 10% and 85% of the total small intestine length to separate duodenum, jejunum and ileum, respectively, and the weight of each intestinal segment was recorded. Sections (1-2 cm) of each intestinal segment were fixed in Bouin's solutions or snap frozen in liquid nitrogen. The remaining intestinal segments were opened longitudinally and mucosa were collected from duodenum, jejunum and ileum by gently scraping the luminal surface with a sterile microscope slide and snap frozen in liquid nitrogen.

### *Intestinal Histomorphology*

Bouin's-fixed sections of duodenum, jejunum and ileum were embedded in paraffin, sliced to approximately 5  $\mu\text{m}$  with a microtome, and mounted on glass microscope slides and stained with hematoxylin and eosin. Images were captured using a Nanozoomer Digital Pathology System (Hamamatsu Corporation, Bridgewater, NJ) at 10 $\times$  fields. Villus length, villus surface area, and crypt depth were measured in 6-8 well-oriented villi and crypts using the software AxioVision Rel. 4.8 (Carle Zeiss International, Germany). Villus surface area was assessed by outlining the outer surface of the villi within the AxioVision program which then calculated the surface area of the villi (**Figure 2.1**).

### *Jejunal Enterocyte Proliferation*

Enterocyte proliferation was measured using the Click-iT® EdU assay to detect EdU incorporated into proliferating crypt cells. Detection is based on a click reaction, a copper ( $\text{Cu}^{+1}$ ) catalyzed covalent reaction between an azide and an alkyne. In this application, the EdU contains the alkyne and the Alexa Fluor® dye contains the azide. The small size of the dye azide allows

for efficient detection of the incorporated EdU using mild conditions. Standard aldehyde-based fixation and detergent permeabilization are sufficient for the Click-iT® detection reagent to gain access to the DNA. This is in contrast to BrdU (bromodeoxyuridine) assays that require DNA denaturation (typically using HCl or heat or digestion with DNase) to expose the BrdU so that it may be detected with an anti-BrdU antibody (**Figure 2.2**).

In our study, formalin-fixed, paraffin-embedded jejunal samples were sliced to approximately 5 µm with a microtome, mounted on slides, and incubated at room temperature in a humidified chamber with a series of washes with which decreased in the fresh histoclear (National Diagnostics, Atlanta, GA), ethanol, PBS, 0.1% Triton/PBS and 3% bovine serum albumin (BSA)/PBS solution in order to dewax the samples. The ethanol percentage decreased with subsequent washes (100%, 70%, 50%, 25%), To detect cells labeled by EdU, the slides were stained with Alexa Fluor® 555 azide using the Click-iT® EdU Alexa Fluor® 555 Imaging Kits (Invitrogen, Molecular Probes) according to manufacturer's instructions. Total nuclei were counterstained with DAPI (4', 6-diamidino-2-phenylindole dihydrochloride; Invitrogen, Molecular Probes). Approximately 6-8 randomly chosen crypt images per animal was captured using Zeiss LSM 700 confocal microscope (Carl Zeiss International) at 20× fields. Images were qualified based on color density by using the software AxioVision Rel. 4.8 (Carle Zeiss International, Germany). Proliferation was expressed as the percentage of EdU-labeled area divided by totally labeled area per crypt.

#### *Disaccharidase Activity*

Mucosal homogenates were prepared with 0.2 g intestinal mucosa into 2 ml of homogenization buffer containing protease inhibitors (0.45 mol/L sodium chloride, 0.002 mol/L

iodoacetic acid and 0.001 mol/L phenylmethylsulfonylfluoride [Sigma Chemical Co., St. Louis, MO]). After preparing the appropriate dilution in homogenization buffer (range of 0 to 1:20), the mucosal homogenates were incubated in either lactose or sucrose buffer for 60 min at 37 °C. The hydrolysis of the disaccharide by the action of the disaccharidase in the tissue homogenate was stopped by the addition of 2.0% zinc sulfate and 1.8% barium hydroxide. Following centrifugation and transfer of supernatant to a 96-well microplate, the amount of glucose released was detected using a glucose oxidase reagent (Thermo Scientific, Middletown, VA) using a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). Disaccharidase activity was expressed as the amount of glucose released per gram of protein per minute (Dudley et al., 1992).

#### *Mucosal Protein and DNA content*

Mucosal homogenates were prepared as described above. For protein content, homogenates were diluted either 1:5 or 1:10 in homogenization buffer and transferred to a 96-well microplate. Bovine serum albumin (BSA, Sigma Chemical Co.) was used to establish the standard curve. Following the addition of Bradford dye reagent, protein concentration was measured using a spectrophotometer (Molecular Devices). For DNA content, homogenates were diluted 1:5 in 1x Tris-EDTA buffer solution and transferred to 96-well microplate, where samples were incubated with PicoGreen dsDNA quantitation reagent solution (Invitrogen, Molecular Probes, Eugene, OR) and measured using a fluorometer (Molecular Devices). Calf thymus DNA (Sigma Chemical Co.) was used to generate the DNA standard curve.



### *Statistical Analyses*

All data are expressed as mean  $\pm$  standard error of the mean. A one-way ANOVA was performed using a General Linear Model (GLM) with a post hoc LSD test using Fisher's Least Significant Difference test to evaluate the effects of diet (Formula, Formula with OPN or sow milk). Normality of data was tested under Proc Univariate, and homogeneity of variance under the GLM procedure. A Wilcoxon non-parametric test was used under the GLM procedure if normality and/or homogeneity of variance were violated; data were expressed as Chi square value. Analyses were performed using SAS Version 9.2 (SAS Institute, Cary, NC). Statistical significance was assigned at  $P < 0.05$ , with a trend reported as  $P < 0.10$ .

## **Results**

### *Formula Intake and Body Weight*

Formula intake between d2 and d21 postpartum was not significantly different between FF and OPN groups ( $p=0.3678$ ). The mean formula intake of FF group and OPN group was  $1077 \pm 52$  ml/day and  $1154 \pm 65$  ml/day, respectively. Therefore, daily intake of OPN is 44.2 mg/kg BW. The mean birth weight was similar among the three groups ( $1.28 \pm 0.27$  kg,  $p=0.3809$ ). During the first 7 days, body weight tended to differ ( $p=0.0504$ ); during the 14~21 days, the difference between the groups became significant ( $p=0.0003$ ). Overall, SR piglets gained more weight than FF ( $p<0.0001$ ) and OPN ( $p=0.0003$ ) piglets during the 21-day study. There was no effect of OPN ( $p=0.5689$ ) on body weight gain (**Table 2.1, Figure 2.3**).

### *Intestinal Weight and Length*

Intestinal length was unaffected ( $p=0.8035$ ) by different feeding methods. However, the intestinal weight (g) of SR piglets was lower than FF ( $p=0.0021$ ) and OPN ( $p=0.0151$ ) piglets. Similarly, when normalized by body weight, the intestinal weight (g/kg) and intestinal length (cm/kg) of SR piglets were lower than FF ( $p<0.0001$ ,  $p=0.0002$ ) and OPN ( $p<0.0001$ ,  $p=0.0008$ ) piglets. Intestinal weight normalized by intestinal length (g/cm) was also lower in SR than formula ( $p=0.0004$ ) or OPN ( $p=0.0120$ ) piglets. There was no effect of OPN ( $p=0.2466$ ,  $p=0.7417$ ,  $p=0.2194$ ) on any of the above measurements (**Table 2.1**).

### *Mucosal Protein and DNA Content*

Protein. Mucosal protein content of SR piglets was lower than FF ( $p=0.0033$ ) and OPN ( $p=0.0021$ ) piglets in the duodenum. In the jejunum, there was no significant effect of diet among the three groups. Mucosal protein content of SR piglets was higher than FF ( $p=0.0136$ ) and OPN ( $p=0.0023$ ) piglets in the ileum (**Table 2.2**).

DNA. There was no effect of diet on mucosal DNA content in the duodenum ( $p=0.2932$ ), jejunum ( $p=0.9749$ ) and ileum ( $p=0.2777$ ) among the three groups (**Table 2.2**).

### *Intestinal Histomorphology*

In the duodenum, the villus length and villus area of SR piglets were lower than FF ( $p=0.0051$ ,  $p=0.0008$ ) and OPN ( $p=0.0084$ ,  $p=0.0006$ ) piglets. There was no effect of OPN ( $p=0.9254$ ,  $p=0.8372$ ) on any of the above measurements (**Table 2.3**). OPN piglets had deeper ( $p=0.0013$ ) crypts than SR piglets and tended ( $p=0.0928$ ) to have deeper crypts than FF piglets. In the jejunum, no significant difference was observed among the three groups in the villus

length, villus area, and crypt depth. In the ileum, the villus length and villus area of SR piglets were lower than FF ( $p<0.0001$ ,  $p<0.0001$ ) and OPN ( $p=0.0002$ ,  $p<0.0001$ ) piglets. Crypt depth did not differ among three groups in the ileum ( $p=0.4147$ ). There was no effect of OPN ( $p=0.7480$ ,  $p=0.3809$ ,  $p=0.6624$ ) on any morphological outcome in the ileum (**Table 2.3, Figure 2.4 A, B, C**).

#### *Jejunal Enterocyte Proliferation*

There was a significant effect of diet ( $P<0.0001$ ) on jejunal enterocyte proliferation. EdU-labeled area (%) per crypt significantly differed among SR ( $53.5\pm2.0\%$ ), OPN ( $41.6\pm1.9\%$ ) and FF ( $28.6\pm1.7\%$ ) groups. Therefore, DNA synthesis was nearly 2-fold higher in SR piglets compared with that in FF piglets, whereas OPN supplementation reduced this difference by 40% from about 2 folds to 1.2 folds (**Figure 2.5**).

#### *Disaccharidase Activity*

Lactase Activity. Lactase activity reached the highest levels in the jejunum of each group ( $P<0.0001$ ). Duodenal lactase activity ( $\mu\text{mol glucose/min/g protein}$ ) of SR piglets was higher than FF ( $p=0.0041$ ) and OPN ( $p=0.0002$ ) piglets. FF piglets had higher ( $P=0.0075$ ) jejunal lactase activity than SR piglets did. OPN piglets tended to have lower jejunal lactase activity than FF piglets ( $P=0.0597$ ), but no difference was observed between OPN and SR groups ( $p=0.2610$ ). In contrast, lactase activity of SR piglets was lower than FF ( $p=0.0002$ ) and OPN ( $p=0.0002$ ) piglets in the ileum. No effect of OPN on lactase activity was observed in the duodenum ( $p=0.1918$ ) or ileum ( $p=0.9574$ ) (**Table 2.4, Figure 2.6**).

Sucrase Activity. Sucrase activity reached the highest levels in the jejunum of each group ( $P < 0.0001$ ). Duodenal sucrase activity ( $\mu\text{mol glucose/min/g protein}$ ) of OPN piglets did not differ from FF ( $p = 0.3751$ ) or SR ( $p = 0.0671$ ) piglets. However, SR piglets had higher sucrase activity than FF ( $p = 0.0113$ ) in the duodenum, but lower sucrase activity than FF ( $p = 0.0255$ ) and OPN ( $p = 0.0018$ ) piglets in the ileum. In the jejunum, no significant difference in sucrase activity was noted among three groups. In summary, no effect of OPN on sucrase activity was identified in the duodenum, jejunum and ileum (**Table 2.4, Figure 2.6**).

## Discussion

Osteopontin is a highly phosphorylated and glycosylated acidic protein that is present in bovine (Bayless et al., 1997; Sorensen et al., 1995) and human (Nagatomo et al., 2004; Sorensen et al., 2003) milks, but is present at a very low concentration in infant formulas. Methods have been developed to isolate OPN from bovine milk (Bayless et al., 1997), which creates the opportunity to supplement it to human infant formula. Milk-borne OPN may be important in pediatric nutrition for a variety of reasons. It is a multifunctional protein that is involved in both physiologic and pathophysiologic events, including the regulation of cell migration and proliferation (Sodek et al., 2006), tissue repair and angiogenesis (Liaw et al., 1998), cellular chemotaxis (Giachelli et al., 1998) and activation in inflammatory conditions (Ashkar et al., 2000; Hwang et al., 1994).

Despite its presence at relatively high concentrations in human milk and wide-ranging biological activities, little attention is focused on its effects on developmental ontogeny of the digestive functions. Previous research on neonatal Rhesus monkeys demonstrated that the

supplementation of OPN with infant formula regulated jejunal gene expression (Donovan et al., 2011). The neonatal piglet is similar to the human infant with regard to the intestinal structure and digestive enzyme development (Aumaitre et al, 1978; Moughan et al., 1972), making it an ideal model to study the potential role of milk-borne bioactive protein in the neonate. Therefore, the goal of this study was to perform preclinical trials on neonatal piglets in order to determine the impact of bovine OPN on the gastrointestinal structural growth and functional development.

Based on the formula intake of the OPN piglets, they consumed 44.2 mg/kg BW/d. Since the OPN concentration of the formula was in a range of OPN concentration in human milk, daily exposure to OPN in milk would be similar to human infants. Previous studies reported that the mean plasma concentration of 3-month-old breast-fed infants (352 ng/ml) was approximately 10-fold higher than that of adult serum (35 ng/ml) (Schack et al., 2009), suggesting either enhanced endogenous production of OPN in the infant vs. adult or potentially absorption of milk-borne OPN. Unfortunately, at the present time, there is no antibody that recognizes porcine OPN. Therefore, we are unable to measure the concentrations of OPN in either sow milk or piglet serum, thus, OPN intake of the SR piglets or whether serum OPN concentrations were higher in OPN compared to FF piglets could not be assessed.

Our first aim was to investigate the effects of different dietary treatments on body and intestinal structural growth. In this study, SR piglets gained more weight than FF and OPN piglets. We do not feel that this was due to any detrimental effect of formula feeding or OPN on normal growth and development of piglets. Rather, it likely reflects the improved lactation efficiency of the newer breed of swine that were introduced recently at the University of Illinois Swine Research Center. In the past, formula-fed piglets consuming 360 ml/kg/d demonstrated growth rates that were similar to those of SR piglets in litters of 10 (Correa-Matos et al., 2003;

Houle et al., 1997). However, recent studies in our laboratory with the new line of piglets have demonstrated superior growth to SR piglets in litters of 10, compared to FF littermates. Moreover, protein content (5%) in our sow milk replacer was much lower than that in sow colostrum, but was comparable to sow milk. Fat content (2.6%) was consistently lower than sow colostrum and milk (>5%; Klobasa et al., 1987). Therefore, it is very likely that the higher concentration of fat in sow colostrum and milk developed more body weight in the form of subcutaneous and/or visceral fat in neonatal piglets, which was obviously observed during sample collection. Despite greater overall body weight gain, intestinal weight, either in absolute terms (g) or normalized by body weight (g/kg), of SR piglets compared with FF and OPN piglets, which confirmed that the formula-feeding met the nutrient requirement for intestinal growth. Additionally, in the duodenum, two formula-fed groups had higher levels of mucosal protein content than SR group. The higher ratios of mucosal protein to DNA indicated that accumulation of protein exceeded the rate of DNA synthesis, resulting in the hypertrophy of the enterocytes that lined the intestine. The presence of bigger enterocytes would be consistent with higher villi, larger villus surface areas and deeper crypts observed in both formula-fed groups. However, the apparent trophic effect of formula was not seen in the jejunum and ileum. The SR piglets are included as a “gold standard” reference, however, the most appropriate comparison to determine an effect of added OPN is between the two formula-fed treatment groups.

Measuring DNA synthesis is an accurate method to detect cell proliferation. To determine the effects of oral OPN supplementation on enterocyte proliferation, the incorporation of EdU to the DNA backbone of proliferative cells was examined. Both soluble OPN, which works as a cytokine, and immobilized OPN, which function as an extracellular matrix protein, protect against apoptosis and induce survival and proliferation in several cell types. Several *in*

*vitro* studies have reported that OPN exerts pro-survival and/or proliferative effects on adherent cell types such as smooth muscle cells (Weintraub et al., 2000) and epithelial cells (Elgavish et al., 1998). Liu et al. (2009) reported that the overexpression of OPN in human embryo kidney-293 cells significantly increased the level of cell proliferation *in vitro*. Furthermore, OPN's anti-apoptotic and proliferative effects are mostly associated with tumorigenesis. It has been previously indicated that epidermal growth factor (EGF)-dependent proliferation of prostate cancer cells was amplified by OPN (Angelucci et al., 2004). To our knowledge, this study is the first to demonstrate the proliferative effects of OPN on the small intestinal epithelial cells *in vivo*. The significant higher jejunal enterocyte proliferation was noted in SR (53.5%) and OPN (41.6%) piglets, compared with FF piglets (28.6%). Although higher levels of DNA synthesis observed in SR and OPN piglets, there were no effects of dietary treatments on villus height and villus area, suggesting that OPN was promoting intestinal growth at a cellular level. It was previously reported that crypt cell proliferation was markedly increased in SR piglets in the first two days of life with a higher ratio of mitotic activity / apoptosis. (Zabielski et al., 2008) In contrast, this ratio was decreased on day 7 and week 12 (Zabielski et al., 2008). Therefore, although higher rate of enterocyte proliferation was noted in the jejunum, it is possible that higher apoptosis rate also occurred in this section. This developmental pattern was also consistent with the study in which there were little further increases seen in the small intestine of rats after infusion of EGF for longer periods, although EGF significantly increased enterocyte proliferation and crypt and villus area after 24 hours of EGF infusion (Berlanga-Acosta, et al., 2001). There was a tendency ( $P=0.065$ ) for OPN piglets to have deeper crypts than SR piglets did in the jejunum, which may support the effects of OPN on enhancing the proliferation of enterocytes residing in crypts.

Our second aim was to measure digestive enzyme activities to evaluate the effects of OPN on the functional capacity of the intestine. We observed that FF piglets had higher jejunal lactase activity than SR piglets. The jejunal lactase activity of OPN piglets tended to be lower than that of FF piglets, but no difference was seen between OPN and SR piglets. However, this effect was not seen in the jejunal sucrase activity of OPN piglets. The mechanism underlying the tendency of the decrease in jejunal lactase in response to OPN supplementation remains to be elucidated, but could involve direct modulation of lactase gene expression or the posttranslational processing of the enzyme (Burrin et al., 1994). In addition, the results from gene microarray analysis in Rhesus monkeys studies (Donovan et al. 2011) demonstrated that galactose metabolism was one of the main differences in signaling pathways between FF and OPN groups. An earlier study (Smith et al., 1990) reported that galactose inhibited lactase expression by mouse jejunal enterocytes. Therefore, it is possible that different galactose metabolism may play a role in the decrease of lactase activity seen in OPN piglets. Furthermore, higher concentration of lactose (8.8%) in our sow milk replacer formula vs. sow milk (3.1~5.8% during 0 h ~ 21d; Klobasa et al., 1987) may attribute to the increase in lactase activity seen in FF piglets (Marion et al., 2005). SR piglets had higher duodenal lactase and sucrase activities than FF and OPN piglets, suggesting that formula-feeding reduced the lactose digestive capacity, specifically in the proximal part of the small intestine (Thymann et al., 2006). In contrast, SR piglets had lower lactase and sucrase activities than the other two formula-fed groups in the ileum. One possible explanation is that the lactose absorption in the proximal region of the small intestine of SR piglets are more efficient compared with the counterpart in FF and OPN piglets. Less lactose reached the ileum and, thus, less lactase activity was developed in this region. However, considering less lactose hydrolysis in the proximal section, relatively higher



concentrations of lactose may be responsible for the increase in the ileal lactase activity of two formula-fed groups. Taken together, our data suggests that sow milk and piglet formula may have differentiated effects on the development of the disaccharidase activities in the three sections of small intestine in neonatal piglets. In addition to the impacts of dietary treatments across the groups, a typical developmental trend was also observed in our study. The jejunum is the most active region of the intestine for digestion and absorption of dietary components. Thus, it was shown in our study that both lactase and sucrase activities reached the highest levels in the jejunum, compared with their counterparts in the duodenum and ileum, regardless of treatment groups. The decrease in lactase activity in the distal region of the small intestine was in agreement with the previous findings (Manners et al., 1972; Houle et al., 1997; Oliver et al., 2002).

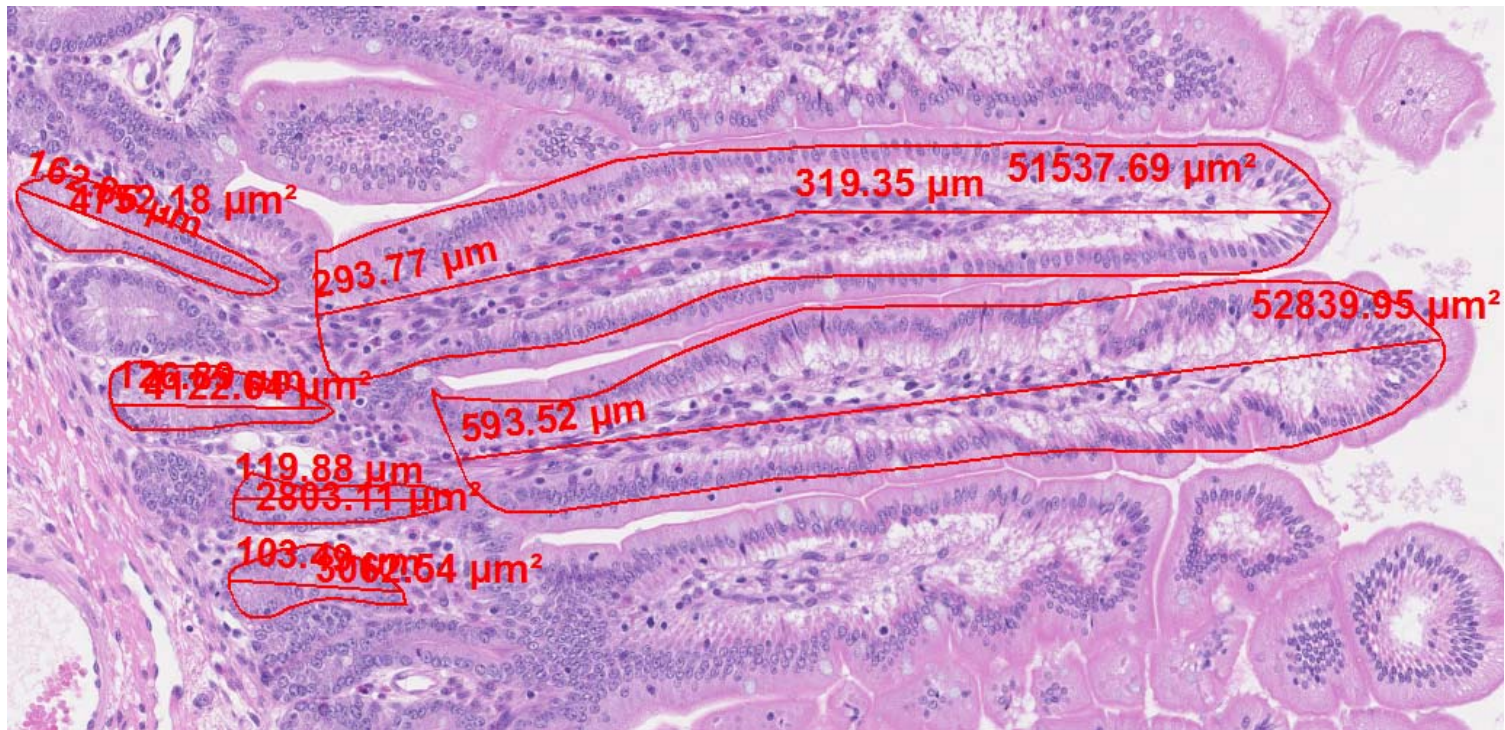
OPN has been recognized as an important luminal regulator (Brown et al., 1992), due to its expression by epithelial cells covering luminal cavities capable of active secretion and absorption of nutrients. Indeed, earlier studies showing that epithelial cells secrete OPN first indicated that OPN is involved in controlling epithelial barrier permeability and secretory functions (Butler, 1989). Recent studies suggested that the constitutive expression of OPN by epithelial cells is required for maintaining the epithelial barrier in the intestines (Gassler et al., 2002). In current study, the effects of OPN on intestinal morphology and digestive enzyme activities were minimal. However, it is very possible that OPN may have effects in the intestine that were not assessed herein. OPN exhibits pro-survival and anti-apoptotic properties, and thus aids cell survival, wound healing and matrix formation. In a previous study of preterm piglets, OPN supplementation in formula was associated with a modest decrease in necrotising enterocolitis (NEC) without any significant effects on gut structural and functional indices

(Møller et al., 2011). In that study, daily intake of OPN in preterm piglets was 6-fold higher than that used in our study. Therefore, further studies are needed to determine the effects of bovine OPN at different concentrations on the small intestinal growth and functional development in disease or infection model of piglets. Furthermore, a RGD cell-binding domain in OPN suggests that this protein might play a role in cell adhesion (Oldberg et al., 1986), which is a critically important for maintaining barrier function in the intestine. Osteopontin expressed by breast epithelium and purified from human milk promoted attachment and spreading of a variety of cell types, including epithelial cells (Brown et al., 1992) and behaved in similar fashion as adhesive proteins. A likely hypothesis is that OPN binds luminal epithelial cell surface integrins via its RGD domain. In addition, the finding that OPN is widely distributed on surfaces of specific populations of epithelial cells in the gastrointestinal tract, bile ducts, pancreatic ducts, urinary tracts and breast suggests that this protein may have a protective role in interactions between these epithelial surfaces and the external environment. Thus, OPN present in breast milk may protect the gastrointestinal epithelium of the nursing baby and the breast epithelium of the mother.

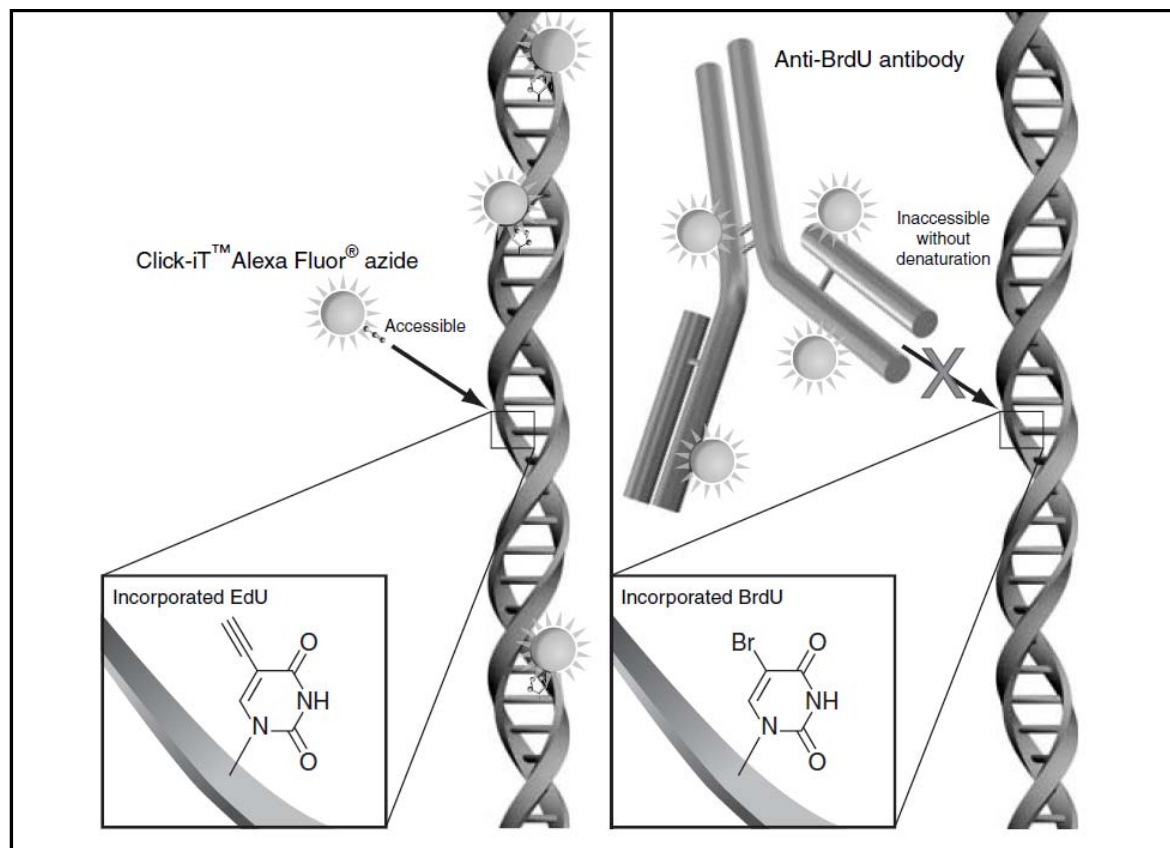
In bovine milk, OPN is present as an intact protein or in several proteolytically generated N- and C-terminal fragments (Sørensen & Petersen, 1993b). Furthermore, bovine milk OPN was partially resistant to proteolysis by neonatal gastric juice (Chatterton et al., 2004). It is not known if all forms of OPN bind equivalently well to their cell surface receptors. However, isolated forms of OPN have been shown to bind to monocytes through a novel integrin receptor *in vitro*, thus enhancing phagocytosis. Given that we observed some differences between OPN and FF piglets, we are hypothesized that ingested OPN is able to bind to epithelial cell surface integrins in the small intestine of piglets. In addition, previous work from our laboratory has

shown that the supplementation of OPN to formula at human milk concentrations decreased the differences in jejunal gene expression between formula-fed and mother-fed Rhesus monkeys (Donovan et al., 2011). Differences in signaling pathways regulating stem cell proliferation (WNT), gastrointestinal patterning (HEDGEHOG) and cell fate between secretory and absorptive lineages (NOTCH) were differentially expressed among the groups. Future studies will assess gene expression in the jejunum of these piglets using gene microarray to determine whether supplemental OPN is also modulating the intestinal transcriptome of piglets.

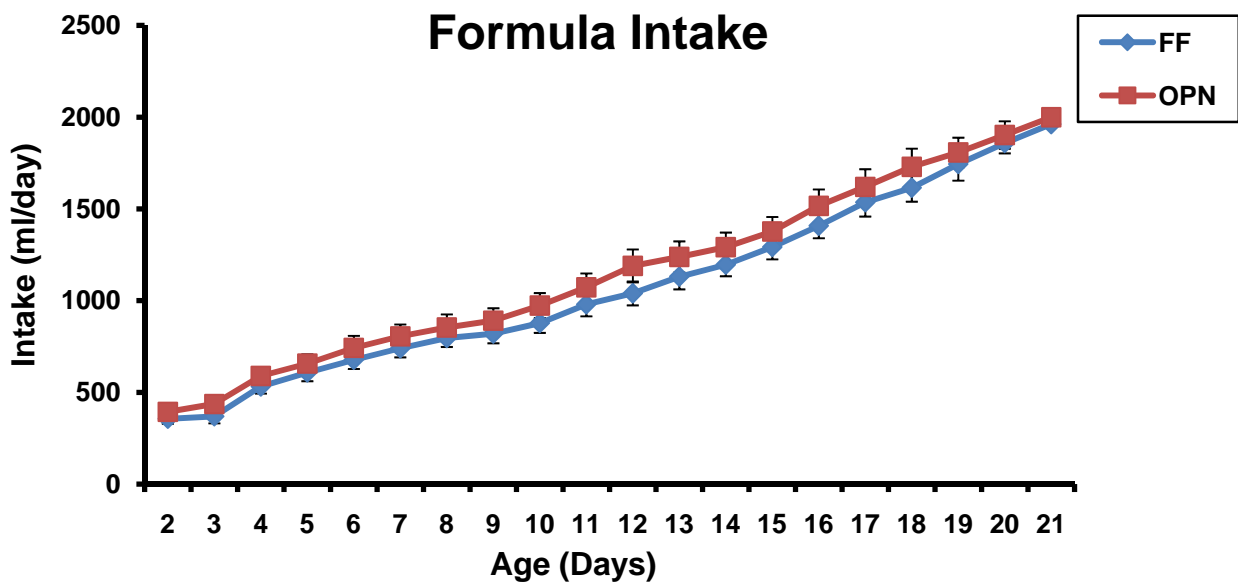
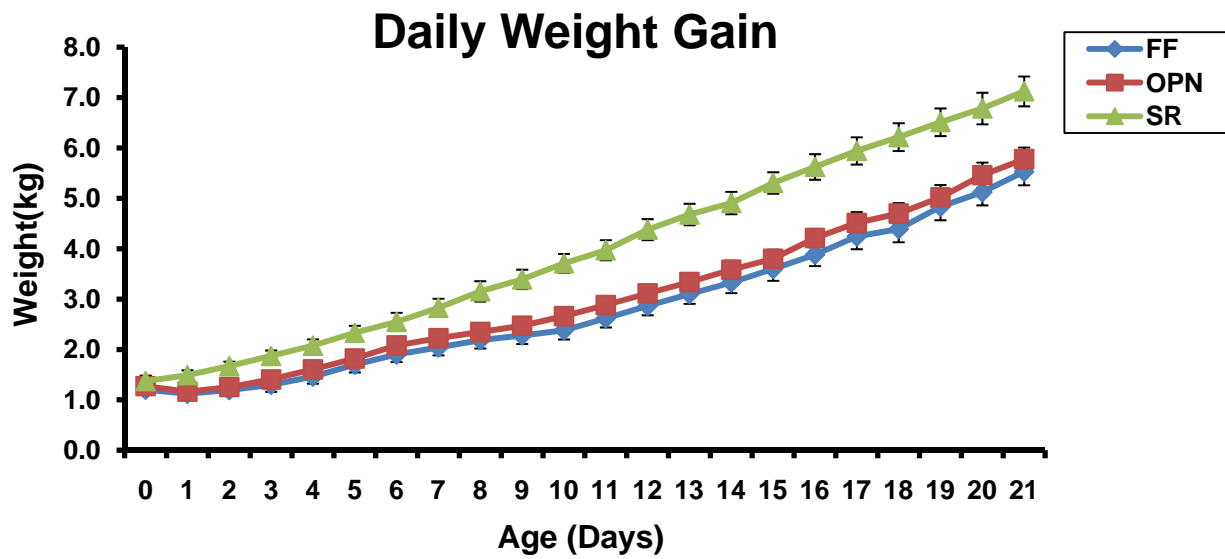
In summary, the addition of OPN in formula increased jejunal crypt cell proliferation, independent of evident morphological growth, and had a minor impact on lactase activity in the small intestine of neonatal piglets. Bovine OPN at human milk concentrations may not significantly affect intestinal structure and functional development in healthy, growing neonatal piglets. Therefore, further studies are needed to determine the functional fragments of bovine OPN and the effects of bovine OPN at different concentrations on the small intestinal growth and functional development in disease or infection model of piglets.



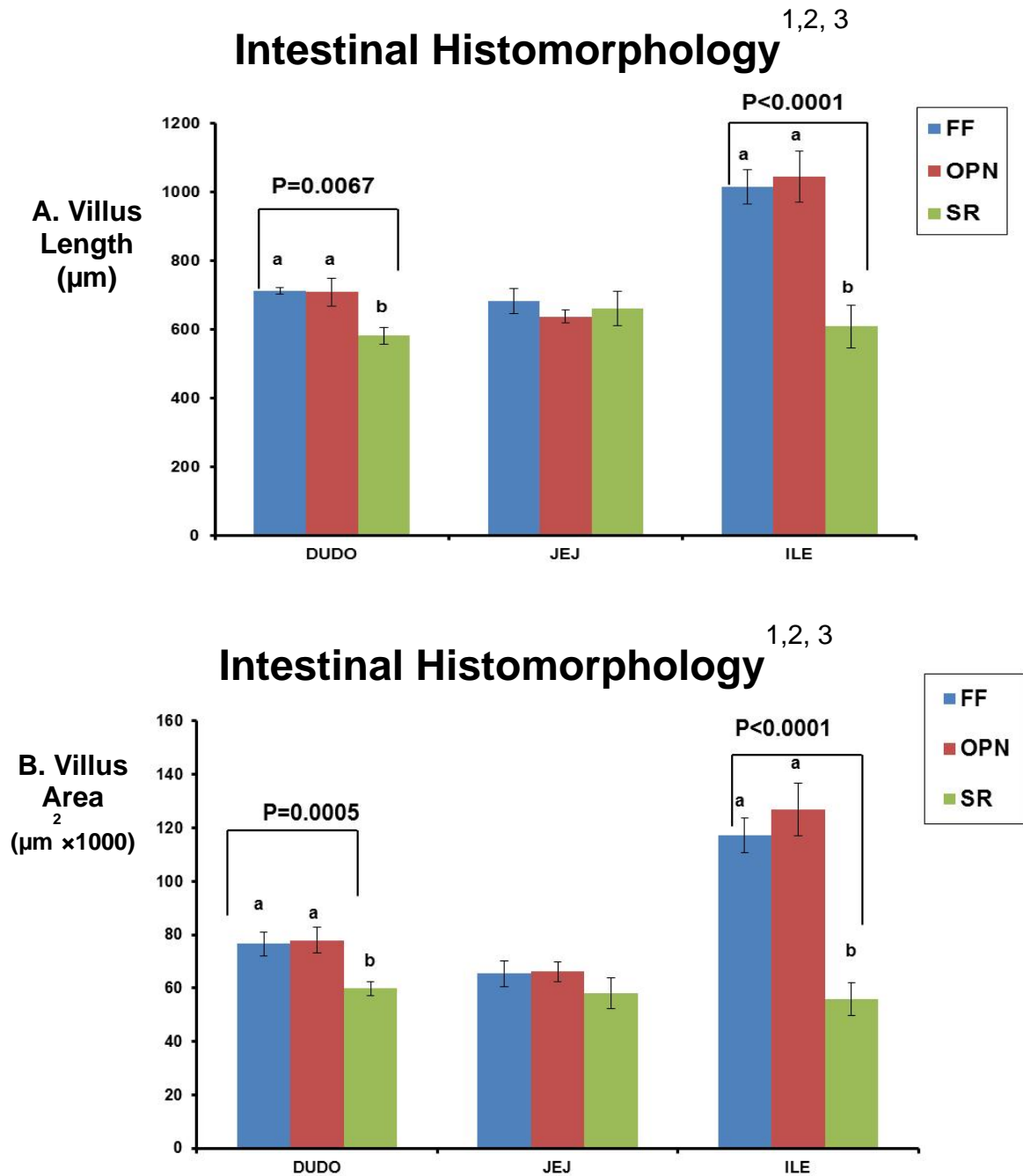
**Figure 2.1 The measurements of intestinal histomorphology.** Images were captured using a Nanozoomer Digital Pathology System (Hamamatsu Corporation, Bridgewater, NJ) at 10 $\times$  fields. Villus length, villus surface area, and crypt depth were measured in 6-8 well-oriented villi and crypts using the software AxioVision Rel. 4.8 (Carle Zeiss International, Germany) (Figure 2.1). Villus surface area was assessed by outlining the outer surface of the villi within the AxioVision program which then calculated the surface area of the villi.



**Figure 2.2 Detection of the incorporated EdU with the Alexa Fluor® azide versus incorporated BrdU with an anti-BrdU antibody.** The small size of the Alexa Fluor® azide eliminates the need to denature the DNA for the EdU detection reagent to gain access to the nucleotide.

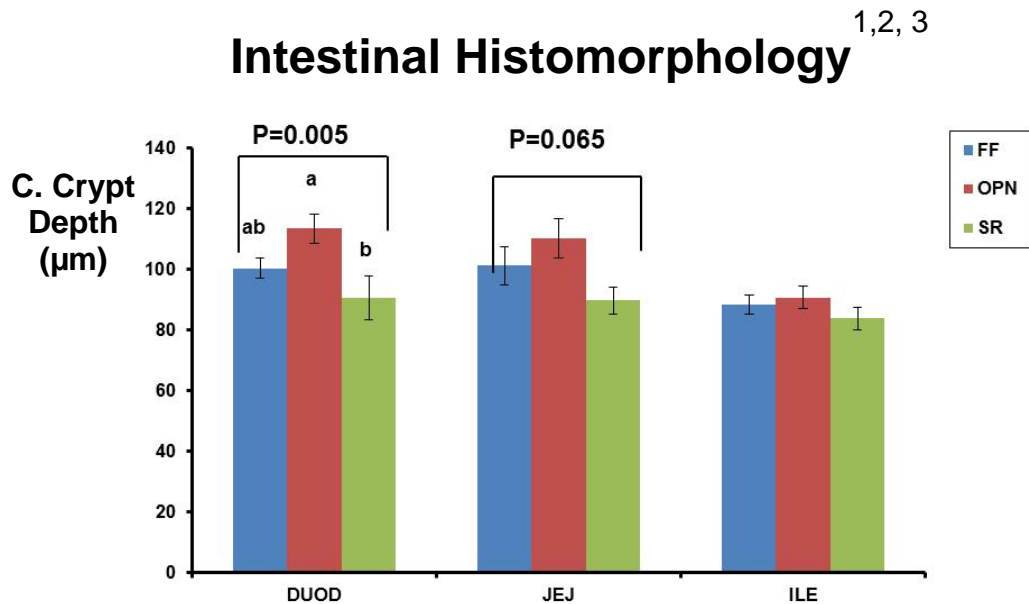


**Figure 2.3 Formula intake and body weight gain.** Overall, SR piglets gained more weight than FF ( $p < 0.0001$ ) and OPN piglets ( $p = 0.0003$ ) during the 21-day study (top panel). Formula intake between d2 and d21pstartum was not significantly different between FF and OPN groups ( $P = 0.3678$ ) (bottom panel). There was no effect of OPN ( $p = 0.5689$ ) on body weight gain. Abbreviations: FF, formula-fed; OPN, formula with OPN; SR, sow-reared.



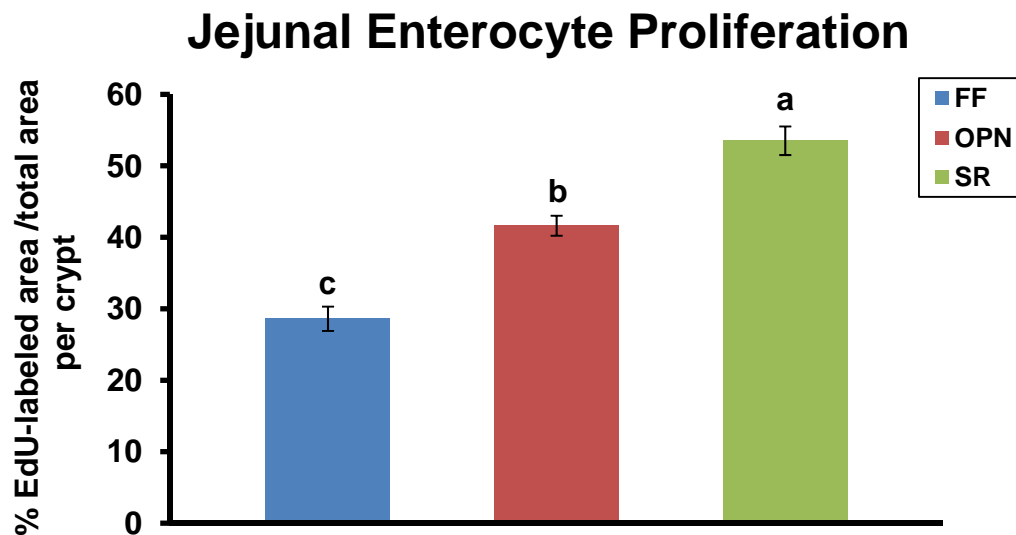
**Figure 2.4A & B Intestinal histomorphology.**

1. FF, formula-fed; OPN, formula with OPN; SR, sow-reared
2. DUOD, duodenum; JEJ, jejunum; ILE, ileum
3. <sup>abc</sup> Values within intestinal sections (DUOD, JEJ or ILE) lacking a common letter differ at  $P<0.05$ .



**Figure 2.4C Intestinal histomorphology**

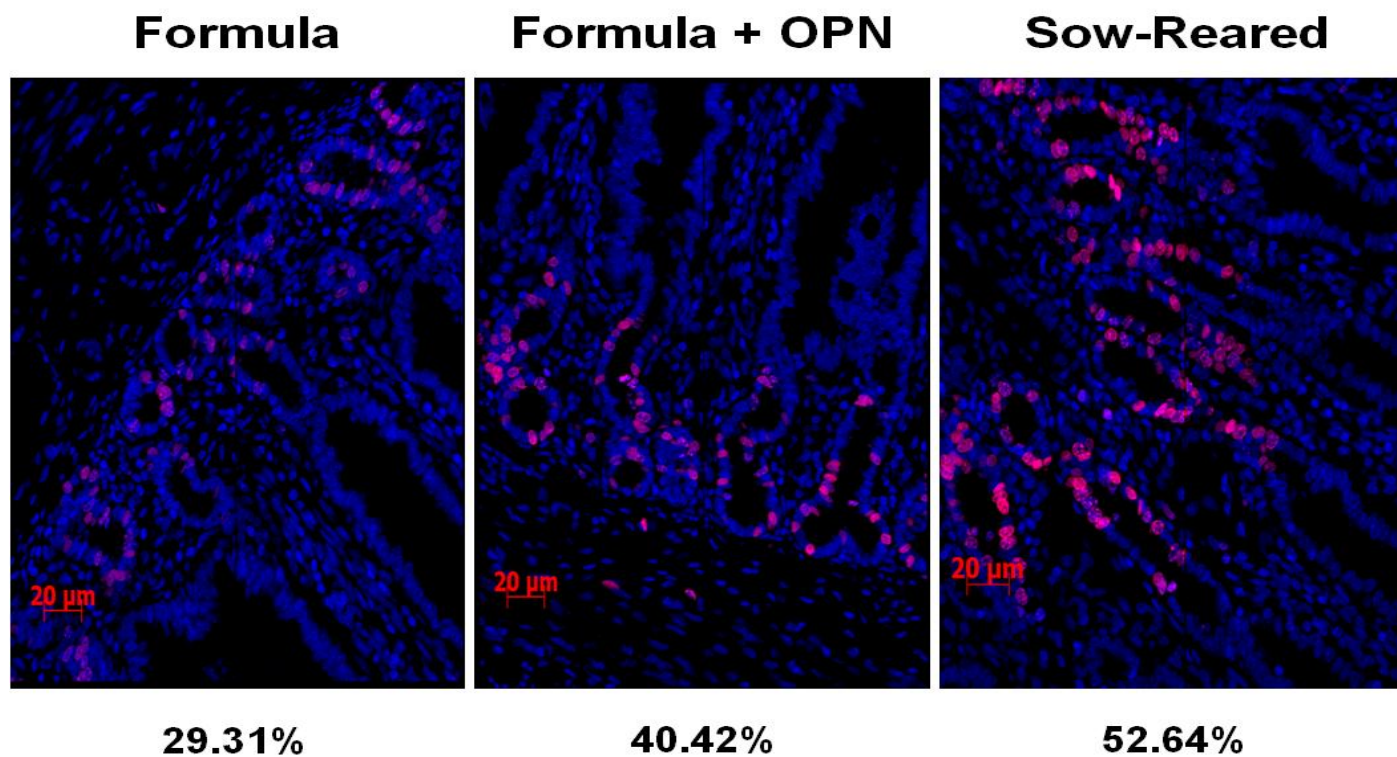
1. FF, formula-fed; OPN, formula with OPN; SR, sow-reared
2. DUOD, duodenum; JEJ, jejunum; ILE, ileum
3. <sup>abc</sup> Values within intestinal sections (DUOD, JEJ or ILE) lacking a common letter differ at  $P < 0.05$ .



**Figure 2.5A Effects of diet on jejunal crypt cell proliferation.** There was a significant effect of diet on jejunal crypt cell proliferation. EdU-labeled area (%) per crypt significantly differed among SR ( $53.5 \pm 2.0\%$ ), OPN ( $41.6 \pm 1.9\%$ ) and FF ( $28.6 \pm 1.7\%$ ) groups. Bars with different letters indicate significant differences between different dietary treatment groups at  $p < 0.0001$ . Abbreviations: FF, formula-fed; OPN, formula with OPN; SR, sow-reared.

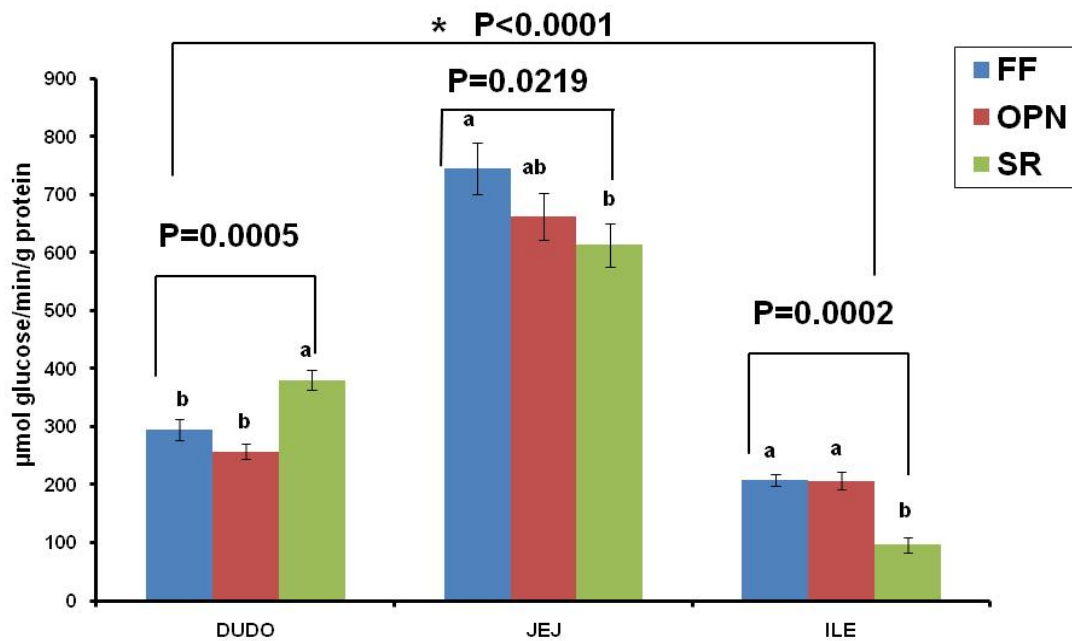


## Jejunal Crypt Cell Proliferation (%) (21-day piglets)

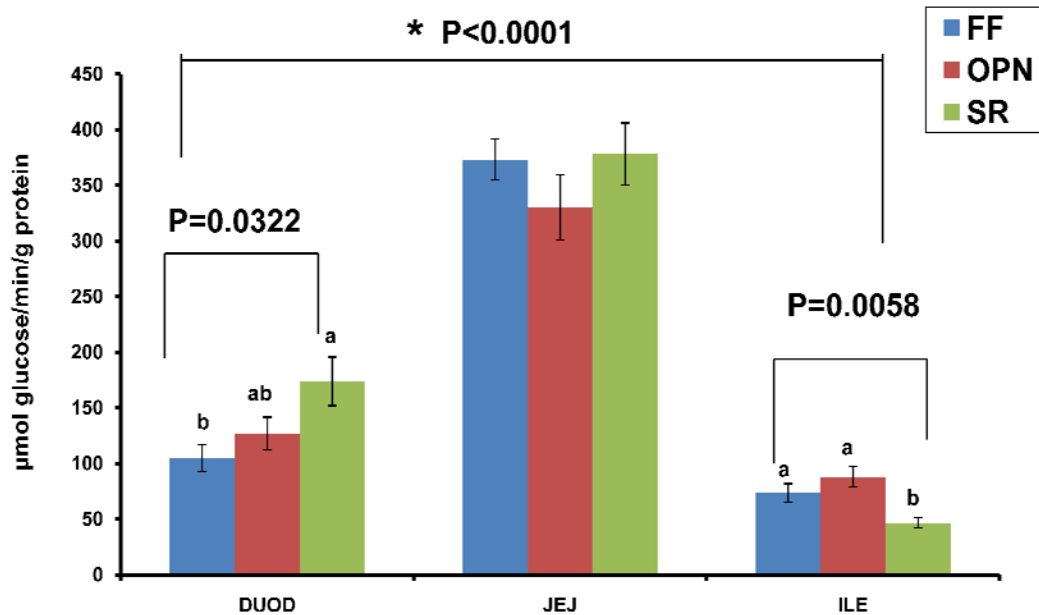


**Figure 2.5B Effects of diet on jejunal crypt cell proliferation.** There was a significant effect ( $p < 0.0001$ ) of diet on jejunal crypt cell proliferation. Abbreviations: FF, formula-fed; OPN, formula with OPN; SR, sow-reared.

## A. Intestinal Lactase Activity



## B. Intestinal Sucrase Activity



**Figure 2.6 Intestinal disaccharidase activity.**

<sup>abc</sup> Values within intestinal sections (DUOD, JEJ or ILE) among groups or

\*values between sections within group lacking a common letter differ at P<0.05.

**Table 2.1 Body weight and intestinal weight and length.**<sup>1,2,3</sup>

	<b>FF</b>	<b>OPN</b>	<b>SR</b>	<b>P value</b>
Body Weight Gain (kg) <sup>4</sup>	4.33 ± 0.18 <sup>b</sup>	4.50 ± 0.24 <sup>b</sup>	5.75 ± 0.20 <sup>a</sup>	<0.0001
Intestinal Length (cm)	801.1 ± 24.63	821.3 ± 35.90	796.5 ± 22.40	0.8035
Intestinal Weight (g)	279.9 ± 13.40 <sup>a</sup>	268.5 ± 10.90 <sup>a</sup>	237.3 ± 5.69 <sup>b</sup>	0.0051
Intestinal Weight/BW (g/kg)	49.6 ± 2.08 <sup>a</sup>	46.2 ± 2.70 <sup>a</sup>	31.61 ± 1.23 <sup>b</sup>	<0.0001
Intestinal Length/BW (g/kg)	142.6 ± 5.70 <sup>a</sup>	140.1 ± 4.20 <sup>a</sup>	111.6 ± 5.30 <sup>b</sup>	<0.0003
Intestinal Weight/Length (g/cm)	0.35 ± 0.01 <sup>a</sup>	0.33 ± 0.01 <sup>a</sup>	0.28 ± 0.01 <sup>b</sup>	0.0012

<sup>1</sup>Data are expressed as mean ± SEM

<sup>2</sup>Abbreviations: FF, formula-fed; OPN, formula with OPN; SR, sow-reared

<sup>3</sup>Means within a row without a common letter differ at p<0.05

<sup>4</sup>Body weight change between d1 and d21 of age

**Table 2.2 Protein and DNA content.**<sup>1,2,3</sup>

	FF	OPN	SR	p-value
<b>Protein (mg/g mucosa)</b>				
DUOD	92.8 ± 3.11 <sup>a</sup>	94.0 ± 1.93 <sup>a</sup>	79.6 ± 3.26 <sup>b</sup>	0.0026
JEJ	83.5 ± 4.21	80.8 ± 2.70	77.6 ± 3.23	0.5061
ILE	68.5 ± 3.54 <sup>b</sup>	64.5 ± 4.16 <sup>b</sup>	80.6 ± 1.79 <sup>a</sup>	0.0056
<b>DNA (µg/g mucosa)</b>				
DUOD	35.6 ± 3.42	28.8 ± 3.83	33.9 ± 1.73	0.2932
JEJ	24.6 ± 3.42	23.9 ± 2.65	23.8 ± 2.35	0.9749
ILE	36.7 ± 4.00	35.0 ± 2.64	42.6 ± 3.53	0.2777

<sup>1</sup> Data are expressed as mean ± SEM

<sup>2</sup> Abbreviations: FF, formula-fed; OPN, formula with OPN; SR, sow-reared; DUOD, duodenum; JEJ, jejunum; ILE, ileum

<sup>3</sup> Means within a row without a common letter differ at  $p < 0.05$

**Table 2.3 Intestinal Histomorphology.**<sup>1,2,3</sup>

	<b>FF</b>	<b>OPN</b>	<b>SR</b>	<b>p-value</b>
<b>Villus Height (µm)</b>				
DUOD	712.4± 9.89 <sup>a</sup>	708.1 ± 39.35 <sup>a</sup>	580.9 ± 25.24 <sup>b</sup>	0.0067
JEJ	682.0 ± 37.29	637.2 ± 18.85	661.0 ± 50.47	0.7564
ILE	1014.4 ± 50.82 <sup>a</sup>	1044.4 ± 75.15 <sup>a</sup>	608.1 ± 61.34 <sup>b</sup>	<0.0001
<b>Villus Area (µm<sup>2</sup>×1000)</b>				
DUOD	76.55 ± 4.59 <sup>a</sup>	77.96 ± 4.86 <sup>a</sup>	59.78 ± 2.67 <sup>b</sup>	0.0005
JEJ	65.34 ± 4.73	66.12 ± 3.69	58.12 ± 5.69	0.4664
ILE	117.2 ± 6.41 <sup>a</sup>	126.9 ± 9.93 <sup>a</sup>	55.79 ± 6.26 <sup>b</sup>	<0.0001
<b>Crypt Depth (µm)</b>				
DUOD	100.3 ± 3.41 <sup>a</sup>	113.4 ± 4.80 <sup>a</sup>	90.6 ± 7.27 <sup>b</sup>	0.005
JEJ	101.2 ± 6.22 <sup>ab</sup>	110.2 ± 6.40 <sup>a</sup>	89.7± 4.34 <sup>b</sup>	0.065
ILE	88.3 ± 3.11	90.7 ± 3.89	83.8 ± 3.68	0.4147

<sup>1</sup> Data are expressed as mean ± SEM

<sup>2</sup> Abbreviations: FF, formula-fed; OPN, formula with OPN; SR, sow-reared; DUOD, duodenum; JEJ, jejunum; ILE, ileum

<sup>3</sup> Means within a row without a common letter differ at p < 0.05

**Table 2.4 Intestinal Disaccharidase Activity.**<sup>1,2,3</sup>

	<b>FF</b>	<b>OPN</b>	<b>SR</b>	<b>p-value</b>
<b>Lactase (μmol glucose/min/g protein)</b>				
DUOD	294.8 ± 18.69 <sup>b</sup>	257.4 ± 13.94 <sup>b</sup>	380.0 ± 17.25 <sup>a</sup>	0.0005
JEJ	645.4 ± 44.23 <sup>a</sup>	562.3 ± 40.56 <sup>ab</sup>	513.6 ± 38.12 <sup>b</sup>	0.0219
ILE	208.1 ± 10.16 <sup>a</sup>	206.9 ± 15.39 <sup>a</sup>	96.6 ± 12.92 <sup>b</sup>	0.0002
<b>Sucrase (μmol glucose/min/g protein)</b>				
DUOD	105.1 ± 12.19 <sup>b</sup>	126.84 ± 14.69 <sup>a,b</sup>	173.7 ± 21.92 <sup>a</sup>	0.0322
JEJ	373.1 ± 18.58	330.1 ± 29.72	378.2 ± 27.86	0.3738
ILE	73.7 ± 8.25 <sup>a</sup>	87.9 ± 9.18 <sup>a</sup>	46.7 ± 4.97 <sup>b</sup>	0.0058

<sup>1</sup> Data are expressed as mean ± SEM

<sup>2</sup> Abbreviations: FF, formula-fed; OPN, formula with OPN; SR, sow-reared; DUOD, duodenum; JEJ, jejunum; ILE, ileum

<sup>3</sup> Means within a row without a common letter differ at  $p < 0.05$

## **Chapter 3**

### **Effect of Osteopontin on Serum Antibody Responses to Rotavirus Vaccination in Neonatal Piglets**

#### **Abstract**

The objective of this study was to investigate whether supplemental osteopontin (OPN) influenced the serum antibody responses to rotavirus vaccination in neonatal piglets. Newborn, colostrum-deprived piglets were randomized into two dietary groups: formula with bovine OPN (OPN; 140 mg/L) and formula alone (FF) for 35 days. On d7, piglets in each dietary group were further randomized to receive rotavirus (RV) vaccination (Rotarix<sup>®</sup>) (FF+RV and OPN+RV) or remained non-vaccinated (FF+NV and OPN+NV). Booster vaccination was provided on d14. Blood samples were collected on d7, 14, 21, 28 and 35. RV-specific serum immunoglobulin (Ig) G, IgA, IgM and total serum IgG, IgA, IgM were measured by ELISA. Statistical significance was assigned at  $P < 0.05$ , with trends reported as  $P < 0.10$ . Body weight gain was unaffected by diet and/or vaccination. No significant effect of oral OPN supplementation was observed for RV-specific antibody responses and total Igs levels. After the combination of dietary groups, RV piglets had significantly higher RV-specific IgM concentrations compared to NV piglets. Although there were higher means of RV-specific IgG and RV-specific IgA concentrations in RV group than their counterparts in NV group, the difference did not reach statistical significance. RV-specific IgM reached a peak at d7 post booster vaccination (PBV), whereas the RV-specific IgG and IgA peaked later at PBV 14 or 21. Total Igs were unaffected by RV vaccination but were significantly changed over time, following similar pattern as RV-specific Igs. In summary, neonatal piglets generated weak antibody responses to RV vaccination.

Supplemental OPN did not enhance RV-specific serum antibody responses and total serum Ig levels in neonatal piglets with or without RV vaccination.

## **Introduction**

Osteopontin (OPN) is a multifunctional protein that is synthesized by a variety of nonimmune and immune cells and is involved in interactions with cells mediating signaling, migration, and attachment (Sodek et al., 2000). Within the immune system, OPN is a cytokine secreted by activated T cells, NK cells, dendritic cells, and macrophages during inflammatory processes (Kawamura et al., 2005; Sodek et al., 2006). Therefore, OPN plays a key role in the development and maintenance of immune responses (Wang and Denhardt, 2008). It has been shown to be an important early regulator of Th1-mediated immunity by inducing secretion of the Th1 cytokine IL-12 and inhibiting the production of the Th2 cytokine IL-10 (Ashkar et al., 2000). Moreover, it has recently shown that OPN acts as an opsonin that enhances bacterial phagocytosis (Schack et al., 2009).

Schack and colleagues developed ELISAs (enzyme-linked immunosorbent assay) specific for OPN and measured its concentration in human milk, bovine milk and select infant formulas (Schack et al., 2009). They demonstrated that the OPN concentration in human milk is considerably higher than in bovine milk and infant formulas and that OPN levels in infant plasma were 10-fold higher than in adults. It was also found that milk OPN was able to induce IL-12 secretion from human lamina propria mononuclear cells (LPMNC) isolated from gut biopsies (Schack et al., 2009).

Rotavirus (RV) is the most common viral cause of severe gastroenteritis in infants and young children worldwide (Parashar et al., 2006). The World Health Organization (WHO)



estimates that rotavirus diarrhea results in approximately half a million deaths and approximately 2.4 million hospitalizations in developing countries each year (WHO, 2007). Osteopontin expression was up-regulated in the intestine of pigs following infection with *Salmonella typhimurium* or *Salmonella choleraesuis* (Burkey et al., 2006), providing support for a role of OPN in mediating host defense within the intestine. Additional findings in the literature using OPN null mice suggest that OPN modulates the gastrointestinal immune response in a dextran sulphate sodium (DSS)-induced colitis model (Da Silva et al., 2006) and following RV infection (Rollo et al., 2005). Taken together, these studies suggest that milk-borne OPN might be involved in host defense mechanisms of RV infection within in the neonate. However, this remains to be proven in appropriate preclinical models and clinical studies.

The determinants of protective active immunity to rotavirus are not well understood, but many reports indicate that serum anti-rotavirus immunoglobulins (Igs), especially IgA, were a marker of protection against rotavirus infection and moderate-to-severe diarrhea (Velazquez et al., 2000; Parreño et al., 1999).

The piglet model has several advantages over other animal models for studies of immunity to human rotavirus (HRV). In comparison with mice and calves, the intestinal tract physiology, body size, type of passive immunity (milk IgA) and immune development of piglets resemble those of human infants more closely. Piglets are susceptible to HRV infection and produce subsequent diarrhea (up to 8 weeks or more after birth), which allows studies of the immune responses and the evaluation of vaccine efficacy with booster doses (Yuan et al., 2002). In addition, pigs are born devoid of maternal antibodies, acquiring serum Igs by intestinal absorption of colostrum Igs for up to 36 h after birth before gut closure (cessation of absorption

of intact Ig) occurs (Banks & McGuire, 1989), facilitating experimental manipulation of levels of maternal antibodies.

Therefore, in our study, the colostrum-deprived piglets were utilized to determine the role of milk-borne OPN in the host antibody responses to HRV vaccination. We hypothesized that OPN supplementation could enhance serum RV-specific antibody responses induced by HRV vaccination. In addition, serum Igs were measured as markers of systemic immune development.

## **Materials and Methods**

### *Chemicals*

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise indicated.

### *Study Design and Dietary treatments*

All animal protocols were approved by the Institutional Animal Care and Use Committee at the University of Illinois. Newborn, colostrum-deprived piglets (n=24) were obtained by Cesarean section of term gestation sows (n=3), weighed, and littermates were randomized into two dietary groups: Formula alone (FF, n=12) or formula with OPN at 140 mg/L (OPN, n=12). Piglets were fed a nutritionally-complete commercial piglet formula (Advance Liquiwean, MSC, Dundee, IL) comprised of 25% protein, 13% fat, 48% lactose, 6.5% ash, and containing levels of vitamins, minerals, and trace elements to meet NRC requirements for piglets (NRC, 1998). To provide passive immunity, all piglets were orally gavaged with pregnant sow serum at the rates of 4ml, 5ml and 10 ml per kg body weight at 3, 12 and 22 hr postpartum, respectively (Sangild et

al, 2002). On d2 postpartum, piglets were administered 1ml of iron dextran (Durvet, Inc Blue Springs, MO) by intramuscular injection. Diets were prepared as 20% solids with the addition of double distilled water in a blender (200g/Liter). The bovine OPN was provided as a dried, purified powder by Arla Foods Ingr. (Vibjy P/S, Denmark). The powder is ~ 83% purified OPN by weight. It was diluted in double distilled water and was added to the formula immediately before feeding at a final concentration of 116 mg/L. Formula was offered at 21 times daily at an average rate of 13.8 ml/kg/h for the first 4 days and 17 ml/kg/h thereafter. Formula intake, stool consistency and activity scores were assessed daily (Correa-Matos et al., 2003). Formula not consumed by the piglet was recorded at each feeding in order to accurately determine the volume of formula consumed daily.

On d7, piglets in each dietary group were further randomized to be vaccinated against rotavirus (FF+RV: n=6; OPN+RV: n=6) or non-vaccinated (FF+NV: n=6 or OPN+NV: n=6). RV piglets were orally given a clinically-approved vaccine (Rotarix<sup>®</sup>, GlaxoSmithKline, Research Triangle Park, NC) at a dose of 1ml/piglet, which is the dose recommended for human infants. Booster vaccination (at the equal dose as the first vaccination) was orally administered on d14.

### *Sample Collection*

Blood samples (2 ml) were collected on d7, 14, 21 and 28 by jugular puncture. The study was terminated at d35. Piglets were sedated with Telazol (Tiletamine HCl and Zolazepam HCl, 3.5mg/kg BW each, Pfizer Animal Health, Fort Dodge, IA) and then euthanized by an intracardiac injection of sodium pentobarbital (Fatal Plus, Vortech Pharmaceuticals, Dearborn,

MI; 72mg/kg body weight). Final blood samples were collected by cardiac puncture into heparin-laced vials and serum was separated by centrifugation.

#### *Assessment of RV-Specific Antibody Responses*

To determine isotype-specific antibody titers to RV vaccination in serum samples, three ELISAs were developed in our laboratory.

RV-Specific Serum IgG ELISA. 96-well ELISA plates were coated with 1:400 dilution of crude OSU RV solution (generously provided by Dr. Theresa Kuhlenschmidt, Department of Pathobiology, College of Veterinary Medicine, University of Illinois) in Carb/Bicarb buffer (pH 9.6) overnight at 4 °C. The assay was completed the next day at room temperature. The next day, plates were blocked with PBS/3% BSA (Sigma, St. Louis, MO) solution for 1 hr followed by washing 3 times with 0.1% Tween 20/PBS. Next, serum samples diluted in 0.5% fish gelatin/PBS were added and incubated for 1 hr. Plates were washed as before. Goat anti-pig IgG conjugated to horseradish peroxidase (HRP, Bethyl Labs, Montgomery, TX) was then added at 1:20,000 dilution and incubated for 1 hr, followed by washing 4-times. TMB (BD Biosciences, San Jose, CA) was added and incubated in the dark for 25 min. Finally, 2N sulfuric acid was added and plates were read at 450nm on a SpectraMax M2e (Molecular Devices, Sunnyvale, CA) with 570 nm plate correction wavelength. RV-specific IgG concentration of each sample was calculated using a standard curve created from stock RV-positive serum. The antibody concentrations for the points on the standard curve were set as the reciprocal of the dilution factor multiplied by 1000. For instance, the 1:100 point on the standard curve is reported as 10.

RV-Specific Serum IgA ELISA. 96-well ELISA plates were coated with 5 µg/ml goat anti-pig IgA (Bethyl Labs) in Carb/Bicarb buffer (pH 9.6) overnight at 4 °C. The assay was

completed the next day at room temperature. Plates were blocked, diluted serum samples were added and incubated as indicated in rotavirus specific IgG ELISA. Biotinylated pig OSU strain RV (see below) at a dilution of 1:1000 in 0.5% fish gelatin/PBS was added and incubated for 1 hr, followed by washing 3 times with 0.1% Tween 20/PBS. Streptavidin-conjugated horse radish peroxidase (S-HRP, R&D systems, Minneapolis, MN) diluted 1:200 in 0.5% fish gelatin/PBS was added and incubated for 1 hr, followed by washing 4 times. Finally, TMB (BD Biosciences) was added and incubated in the dark for 20 min, followed by adding sulfuric acid and reading plates as described in RV-specific IgG ELISA. RV-specific IgA concentration of each sample was calculated using a standard curve created from stock serum. The antibody concentrations for the points on the standard curve were set as described above.

RV-Specific Serum IgM ELISA. 96-well ELISA plates were coated with 10 µg/ml goat anti-pig IgM (Bethyl Labs) in Carb/Bicarb buffer (pH 9.6) overnight at 4 °C. The assay was completed the next day at room temperature. Plates were blocked, diluted serum samples were added and incubated as indicated in rotavirus specific IgG ELISA. Biotinylated pig OSU strain RV (see below) at a dilution of 1:5000 in 0.5% fish gelatin/PBS was added and incubated for 1 hr, followed by washing 3 times with 0.1% Tween 20/PBS. S-HRP (R&D systems) diluted 1:200 in 0.5% fish gelatin/PBS was added and incubated for 1 hr, followed by washing 4-times. Finally, TMB (BD Biosciences) was added and incubated in the dark for 35 min, followed by adding sulfuric acid and reading plates as described in RV-specific IgG ELISA. RV-specific IgM concentration of each sample was calculated using a standard curve created from stock serum. The antibody concentrations for the points on the standard curve were set as described above.

Biotin-labeling of RV-Biotin. Crude OSU RV solution (generously provided by Dr. Theresa Kuhlenschmidt, Department of Pathobiology, College of Veterinary Medicine,

University of Illinois) was first dialyzed against PBS using 3500 molecular weight cutoff dialysis tubing (Fisher Scientific, Pittsburgh, PA). This dialyzed sample was then incubated with 20mM NHS-PEG4-biotin (Pierce, Thermo Fisher Scientific, Rockford, IL) for 2 hr on ice. Biotinylated sample was dialyzed against PBS as above. Resulting RV-biotin was tested by coating a 96-well ELISA plate with the RV-biotin sample and detecting with S-HRP (R&D systems) using TMB (BD Biosciences). Non biotinylated RV was used as negative control.

#### *Total serum immunoglobulin levels*

Total serum immunoglobulin levels were detected by ELISA using commercially-available porcine IgG, IgA, and IgM Quantitation Sets (Bethyl Labs).

Total Serum IgG ELISA. 96-well ELISA plates were coated with 1:100 dilution of Affinity purified Pig IgG (A100-104A) Coating Antibody in Carb/Bicarb buffer (pH 9.6) overnight at 4 °C. The assay was completed the next day at room temperature. Plates were blocked; diluted serum samples were added and incubated as indicated in RV-specific IgG ELISA. The HRP Conjugated Pig IgG Detection Antibody (A100-104P) at a dilution of 1:75,000 in 0.5% fish gelatin/PBS was added and incubated for 1 hr, followed by washing 3 times with 0.1% Tween 20/PBS. Finally, TMB (BD Biosciences) was added and incubated in the dark for 15 min, followed by adding sulfuric acid and reading plates as described in RV-specific IgG ELISA. Total IgG concentration of each sample was calculated using a standard curve created from Pig Reference Serum (RS10-107). Each of standard curve point is listed as 10,000 ng/ml, 500 ng/ml, 250 ng/ml, 125 ng/ml, 62.5 ng/ml, 31.25 ng/ml, 15.6 ng/ml, 7.8 ng/ml and 0 ng/ml (blank).

Total Serum IgA ELISA. 96-well ELISA plates were coated with 1:100 dilution of Affinity purified Pig IgA Coating Antibody (A100-102A) in Carb/Bicarb buffer (pH 9.6) overnight at 4 °C. The assay was completed the next day at room temperature. Plates were

blocked; diluted serum samples were added and incubated as indicated in RV-specific IgG ELISA. The HRP Conjugated Pig IgA Detection Antibody (A100-102P) at a dilution of 1:75,000 in 0.5% fish gelatin/PBS was added and incubated for 1 hr, followed by washing 3 times with 0.1% Tween 20/PBS. Finally, TMB (BD Biosciences) was added and incubated in the dark for 15 min, followed by adding sulfuric acid and reading plates as described in RV-specific IgG ELISA. Total IgA concentration of each sample was calculated using a standard curve created from Pig Reference Serum (RS10-107). Each of standard curve point is listed as 1000 ng/ml, 500 ng/ml, 250 ng/ml, 125 ng/ml, 62.5 ng/ml, 31.25 ng/ml, 15.6 ng/ml, and 0 ng/ml (blank).

Total Serum IgM ELISA. 96-well ELISA plates were coated with 1:100 dilution of Affinity purified Pig IgM Coating Antibody (A100-100A) in Carb/Bicarb buffer (pH 9.6) overnight at 4 °C. The assay was completed the next day at room temperature. Plates were blocked; diluted serum samples were added and incubated as indicated in rotavirus specific IgG ELISA. The HRP Conjugated Pig IgM Detection Antibody (A100-100P) at a dilution of 1:75,000 in 0.5% fish gelatin/PBS was added and incubated for 1 hr, followed by washing 3 times with 0.1% Tween 20/PBS. Finally, TMB (BD Biosciences) was added and incubated in the dark for 15 min, followed by adding sulfuric acid and reading plates as described in rotavirus specific IgG ELISA. Total IgM concentration of each sample was calculated using a standard curve created from Pig Reference Serum (RS10-107). Each of standard curve point is listed as 1000 ng/ml, 500 ng/ml, 250 ng/ml, 125 ng/ml, 62.5 ng/ml, 31.25 ng/ml, 15.6 ng/ml, and 0 ng/ml (blank).

### *Statistical Analyses*

All data are expressed as mean  $\pm$  standard error of the mean. Antibody titers were analyzed using Proc Mixed procedure with repeated measures. In the complete statistical model, the fixed effects were diet, vaccination, time and their interactions. If dietary effect was not significant, the OPN + RV and FF + RV piglets were combined as a single RV vaccination (RV) group; OPN+NV and FF+NV were combined as a single non-vaccinated (NV) group. Post hoc t-tests with Tukey correction were utilized in the event of significant vaccine x time interactions. A one-way ANOVA was used to evaluate the antibody concentrations over time. Analyses were performed using SAS Version 9.2 (SAS Institute, Cary, NC). Statistical significance was assigned at  $P < 0.05$ , with trends reported as  $P < 0.10$ .

## **Results**

### *Mortality Rate*

This was the first RV vaccination study conducted in colostrum-deprived piglet model in our laboratory. Since colostrum-deprived piglets were extremely susceptible to environmental pathogens, 41.6% (10/24) piglets died prior to euthanasia and of those piglets that died, 90% (9/10) died during the first two weeks postpartum. At the termination of the study, blood samples were collected from each of the following groups: FF+RV (n=4), FF+NV (n=2), OPN+RV (n=5) and OPN+NV (n=3).



### *Body Weight Gain*

There was no effect of OPN ( $P=0.71$ ) or vaccination ( $P=0.41$ ) on body weight gain. Since there was no dietary effect, OPN+NV and FF+NV were combined as a single non-vaccinated (NV) group. The average body weight gain for OPN+RV, FF+RV and NV was  $6.80 \pm 0.55$  kg,  $7.18 \pm 0.27$  kg and  $7.48 \pm 0.53$  kg, respectively (**Figure 3.1**).

### *Immunoglobulin Levels in Pregnant Sow Serum*

In order to determine the effects of pre-feeding sow serum in antibody responses, RV-specific Igs and total Igs concentrations were measured in sow serum that was orally-administered to the piglets (**Table 3.1**). RV-specific IgG concentrations (**Table 3.1A**) in sow serum were 8-fold higher compared to the average concentrations of the peak antibody responses (d35) in piglets' serum. However, RV-specific IgA and IgM levels in sow serum were 7% and 15% of the average levels of the peak antibody response (d28 or d21) in piglets' serum, respectively. The relationship among total IgG, total IgA and total IgM was 80%, 2%, 17% of the total Ig concentrations (**Table 3.1B**), which was in the normal range of sow serum Igs levels during early gestation (Klobasa et al., 1985). The total serum IgG percentage was also comparable to its counterpart in sow colostrum (Markowska-Daniel & Pomorska-Mol, 2010).

### *Total Serum Immunoglobulin levels*

In order to determine the dietary and/or vaccination effects on systemic immune development in piglets, serum samples were analyzed for IgG, IgA and IgM concentrations on day 7, day 14, day 21, day 28 and day 35. The average of total serum Igs concentrations from four groups were summarized in **Figure 3.5**. Concentrations of total IgG, IgA and IgM in the

sera of all four treatment groups (FF and OPN, both V and NV) are summarized in **Appendix Tables 1-3, respectively.**

Total Serum IgG. No effect of OPN ( $P=0.71$ ), RV vaccination ( $P=0.07$ ) or OPN  $\times$  RV vaccination ( $P=0.88$ ) was observed in the complete model. The only significant effect was time ( $P=0.0006$ ). After the combination of the dietary groups, the effect of RV vaccination tended to reach statistical significance ( $P=0.05$ ). Between day 7 to day 35, total IgG concentrations in RV group were increased ( $P<0.0001$ ) over time (**Figure 3.2**).

Total Serum IgA. No effect of OPN ( $P=0.85$ ), RV vaccination ( $P=0.31$ ) or OPN  $\times$  RV vaccination ( $P=0.92$ ) was observed in the complete model. The only significant effect was time ( $P<0.0001$ ). After the combination of the dietary groups, the effect of RV vaccination was still not significant ( $P=0.26$ ). Between day 7 to day 35, total IgA concentrations were changed ( $P<0.0001$ ) over time and peaked on day 28 (**Figure 3.3**).

Total Serum IgM. No effect of OPN ( $P=0.73$ ), RV vaccination ( $P=0.13$ ) or OPN  $\times$  RV vaccination ( $P=0.32$ ) was observed in the complete model. The only significant effect was time ( $P<0.0001$ ). After the combination of the dietary groups, the effect of RV vaccination tended to reach statistical significance ( $P=0.10$ ). Between day 7 to day 35, total IgM concentrations were changed ( $P<0.0001$ ) over time and peaked on day 14 (**Figure 3.4**).

### *Assessment of RV-Specific Antibody Responses*

To evaluate the effects of oral OPN supplementation on RV-specific antibody responses, RV-specific IgG, IgA and IgM concentrations were measured on day 7, day 14, day 21, day 28 and day 35. **Antibody Titers of RV-specific IgG, IgA and IgM in the sera of all four treatment groups (FF and OPN, both V and NV) are summarized in Appendix Tables 4-6, respectively.**

RV-Specific Serum IgG. No effect of OPN ( $P=0.82$ ), RV vaccination ( $P=0.35$ ) or OPN  $\times$  RV vaccination ( $P=0.99$ ) was observed in the complete model. The only significant effect was time ( $P<0.0001$ ). Since there was no dietary effect, the OPN + RV and FF + RV piglets were combined as a single rotavirus vaccination (RV) group; OPN+NV and FF+NV were combined as a single non-vaccinated (NV) group (Same below). The effect of RV vaccination was still not significant ( $P=0.29$ ). RV-specific IgG concentrations increased over time in both groups ( $P<0.0001$ ). Between day 7 to day 35, RV piglets had 6.8-fold increase in RV-specific IgG, while NV piglets had 5.3-fold increase in RV-specific IgG (**Figure 3.6**).

RV-Specific Serum IgA. On day 7, RV-specific IgA concentrations were low to undetectable. No effect of OPN ( $P=0.75$ ), RV vaccination ( $P=0.53$ ) or OPN  $\times$  RV vaccination ( $P=0.92$ ) was observed in the complete model. The only significant effect was time ( $P=0.0004$ ). After the combination of the dietary groups, the effect of RV vaccination was still not significant ( $P=0.48$ ). RV-specific IgA concentrations were increased ( $P<0.0001$ ) over time (**Figure 3.7**).

RV-Specific Serum IgM. No effect of OPN ( $P=0.97$ ) or OPN  $\times$  RV vaccination ( $P=0.56$ ) was observed in the complete model. The only significant effect was time ( $P<0.0001$ ). After the combination of the dietary groups, the effects of RV vaccination ( $P=0.02$ ), time ( $P<0.0001$ ) and RV vaccination  $\times$  time ( $P=0.02$ ) were significant. Although 85% d7 piglets had undetectable RV-specific IgM, d14 RV piglets had 5.8-fold higher ( $P=0.0064$ ) RV-specific IgM levels compared to d14 NV piglets. RV-specific IgM titers were changed ( $P<0.0001$ ) over time and peaked on day 21 within each group (**Figure 3.8**).

## Discussion

Milk-borne OPN may be important for pediatric nutrition for a variety of reasons. It is a multifunctional protein that is involved in both physiologic and pathological events, including cell mediated immune responses (Ashkar et al., 2000), chemotaxis of inflammatory cells (Giachelli et al., 1998) and anti-inflammatory responses (Hwang et al., 1994). Concerning the role of OPN in immune responses, it has been described that OPN polarizes the Th-1-related cytokine response and contributes to protective immunity against pathogens, such as herpes simplex virus type 1 and *L. monocytogenes* (Ashkar et al., 2000; Nau et al., 1999). Pabst and colleagues (1989) reported that breast-fed infants showed an increased proliferative response of T-lymphocytes compared to formula-fed infants following vaccination with *Mycobacterium bovis bacillus* Calmette-Guerin (BCG) at birth. A potential role for OPN in mediating host response is corroborated by the observation that BCG grew more rapidly in macrophages derived from OPN-null versus wild-type mice (Nau et al., 1999). Furthermore, an inverse

correlation between tissue OPN expression and disease progression was observed after inoculation with BCG in human subjects (Nau et al., 2000).

Rotaviruses are an important cause of severe acute gastroenteritis, a self-limited illness that may cause fatal volume depletion in young children. The immune response engendered in the host is important to limit future infections, but has limited impact on the acute illness. A variety of other host responses to infection mediate the amelioration of the infection and/or symptoms. It has been shown that increased production and secretion from intestinal epithelial cells of osteopontin occurs in response to infection with rotavirus (Rollo et al., 2005). Serum antibodies are an important and powerful correlate of protection against rotavirus disease (Jiang et al., 2002). Therefore, the objective of this study was to determine the role of OPN in serum antibody response (RV-specific IgG, IgA, IgM) and total Igs levels (total IgG, IgA, IgM) induced by RV vaccination in colostrum-deprived piglets.

Overall, our piglet model (colostrum-deprived, orally serum-gavaged) generated weak antibody response to RV vaccination. The only relatively robust antibody response was shown in RV-specific IgM. At d7 post first vaccination, it was found that RV piglets had significantly higher (5.8-fold) RV-specific serum IgM concentrations compared to NV piglets. At d14 post first vaccination, although RV piglets had higher means of RV-specific IgG (2-fold) and IgA (1.2-fold) concentrations compared with NV piglets, the difference did not reach statistical significance. Because of the small number of piglets available in each group and the degree of variability of vaccination responses in piglets, it was not feasible to statistically evaluate our data. However, the following patterns were evident. RV-specific IgM reached a peak at d7 post booster vaccination (PBV), whereas the RV-specific IgG and IgA peaked later at PBV 14 or 21. This kinetics of RV-specific antibody responses in RV group were consistent with other studies

in which piglets were inoculated with porcine RV or HRV using an ELISPOT assay (Chen et al., 1995; Yuan et al., 1996). Although following the similar pattern, NV group generated weaker RV-specific antibody responses. This suggested a possibility of naturally asymptomatic infection induced by RV in the rearing environment, which is also common in children under two-years-old (Phillips et al., 2010).

Rotarix<sup>®</sup> is a monovalent vaccine composed of an attenuated human rotavirus strain G1P[8]. Three clinical trials involving 5024 infants conducted in Finland, Latin America and Singapore indicated a good immunogenicity of this vaccine (De Vos et al., 2004). Many reports indicate that serum IgA antibodies are important for immunity to rotavirus and resistance to symptomatic re-infection (Yuan et al., 1996; Johansen & Svensson, 1997). In the 3 trials, 61–91% of vaccinated infants developed rotavirus-specific serum IgA antibodies after 2 doses, depending on Rotarix<sup>®</sup> potency (De Vos et al., 2004). In a recent trial conducted in healthy Indian infants, Rotarix<sup>®</sup> increased RV-specific IgA concentrations more than two-fold in 60.5% of infants, while 7.5% of placebo recipients showed a two-fold increase (Narang et al., 2009). However, robust RV-specific IgA response was not observed in our study. In oral rotavirus vaccine trials conducted in humans, higher pre-vaccination antibody titers in serum have been associated with lower rates of seroconversion after vaccination (Friedman et al., 1993; Ward et al., 1997). Parreño et al. (1999) reported that higher titers of circulating maternal antibodies (MatAb) suppressed primary and secondary active antibody response significantly. For a study of the attenuated human RV vaccine/ RV-like particles (VLP) vaccine, there was evidence for suppression of systemic antibody responses by LoMatAb, especially systemic IgA ASC, IgA memory B cells in peripheral blood and serum IgA antibody responses (Nguyen et al., 2006). Thus, the sow serum with relatively low titers of MatAb used in our current study, although

contributing to passive immunity against environmental pathogens in piglets, may impair the development of active immunity and decrease the efficacy of live oral rotavirus vaccines. It is possible that high titers of IgG maternal antibodies in piglets' serum were associated with suppressing primary and secondary responses of RV-specific IgA after inoculation (Parreño et al., 1999).

To gain a perspective of dietary and/or vaccination effects on systemic immune development in piglets, total serum Igs concentrations were analyzed in our study. It was found that total serum IgG levels were much lower than their counterparts in sow-reared (SR) piglets reported in previous research (Bourne et al., 1973). This is not surprising, since our piglets were colostrums-deprived and colostrum is characterized by a high concentration of IgG and relatively lower concentration of IgA and IgM (Markowska-Daniel & Pomorska-Mol, 2010). According to Klobasa's research (1981), the IgA concentrations in the serum of naturally sucking piglets varied in the first 24 hours but then decreased rapidly to their lowest values ( $\approx 200 \mu\text{g/ml}$ ) around 14~28 day of age, whereas the IgM concentrations decreased rapidly following the first 24 hours of life to their lowest levels ( $\sim 600 \mu\text{g/ml}$ ) around 8~14 day of age. In the contrast, our study showed that IgA concentrations peaked at day 28 of age and 4.5-fold (RV group) or 3.4-fold (NV group) higher than their counterparts in naturally sucking piglets. Furthermore, IgM concentrations peaked at day 14 of age and were 1.7-fold (RV group) or 1.3-fold (NV group) higher than their counterparts in naturally sucking piglets. This phenomenon suggests a possible RV vaccination responses and/or naturally asymptomatic RV infection.

Piglet's antibody responses to RV vaccination varied widely in our study. At d7 post first vaccination, only 66% and 44% of RV piglets showed RV-specific IgG and RV-specific IgA responses. Piglets' immune responses to a live oral vaccine may be influenced by the amount of

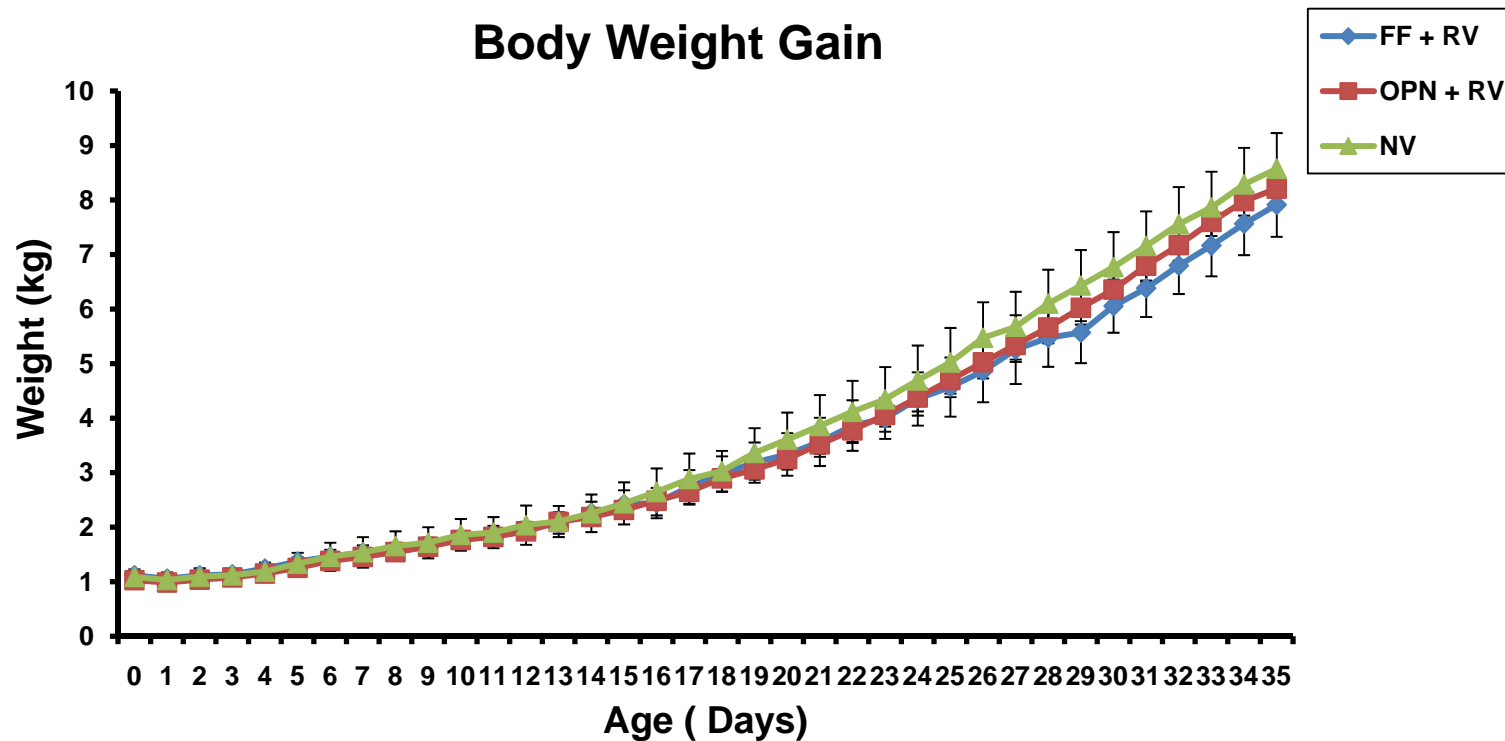
gastric acid in the piglets' digestive tract. Rotavirus can be damaged by low pH in the stomach, and each of the vaccines is administered with a buffer solution to neutralize stomach acid and maintain the titer of the virus. Considering the large discrepancy in the effectiveness of rotarix in infants among developed and developing countries (Patel et al., 2009), it is very likely that a difference in levels of gastric acidity might have influence on vaccine uptake and inducing immune responses. Furthermore, the booster vaccination triggered much lower RV-specific IgA (54%) and IgM (44%) response than the first vaccination did. Nguyen et al. (2005) demonstrated in a RV vaccine study of gnotobiotic piglets that oral priming and intranasal boosting strategies were highly effective in inducing antibody responses. This strategy of avoiding mucosal sites where booster vaccines may be neutralized by local antibodies induced by the priming vaccine assures that booster vaccination will evoke effective antigenic stimulation. Piglets were also received 5ml of 100  $\mu$ M sodium bicarbonate to reduce stomach acidity followed by the oral vaccines.

In the present study, supplemental OPN had no effect on the improvement of antibody responses and total Igs levels in neonatal piglets after RV vaccination. Therefore, the role of OPN in RV vaccination is still inconclusive. However, there is increasing evidence that this protein is involved in many aspects of intestinal physiology in health and disease. Since the existence of OPN in breast milk as well as intestinal epithelial cells (Sodek et al., 2000), OPN in the intestinal lumens of suckling mice consists of OPN in breast milk and from intestinal epithelial cells. The constitutive expression of OPN by epithelial cells is required for maintaining the epithelial barrier in the intestines (Gassler et al., 2002). OPN also has the potential to regulate specific functions of epithelial cells involved in the barrier defense process (Sodek et al., 2006). OPN, moreover, is recognized by  $\alpha_v\beta_3$  and  $\alpha_4\beta_1$  integrins as ligands of the cell adhesion domain

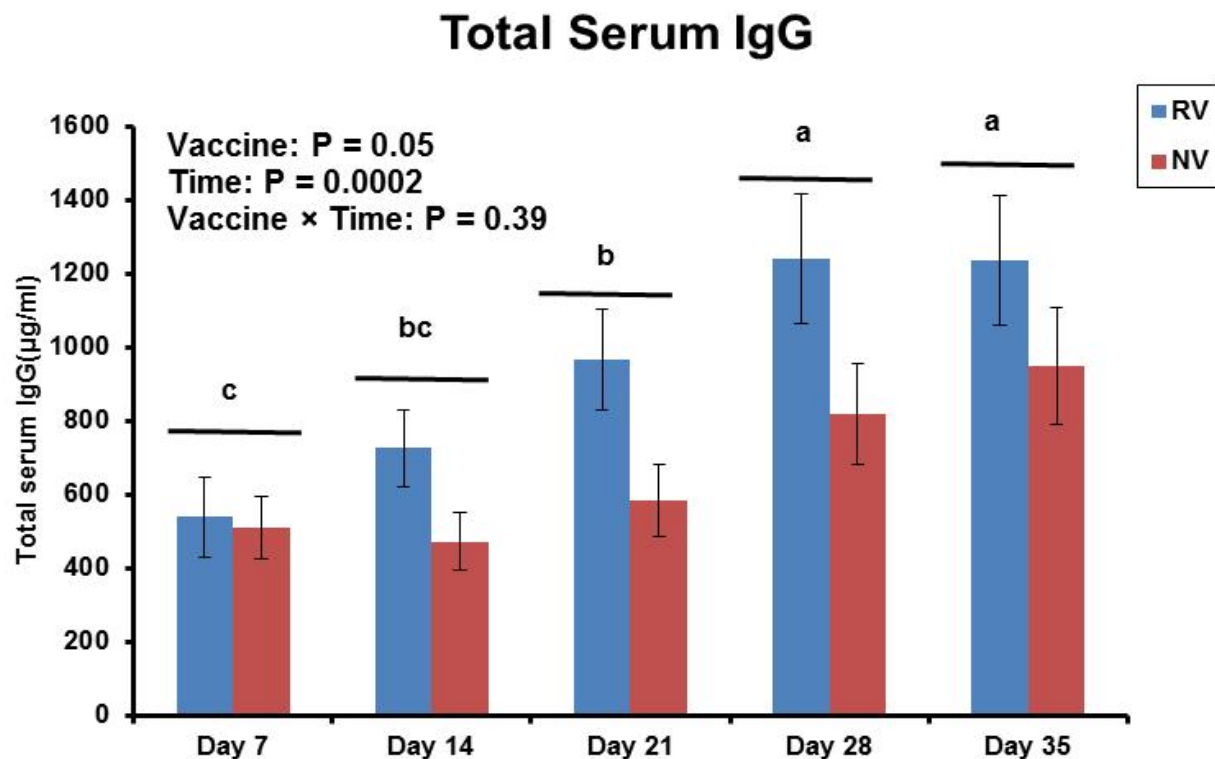


(Bayless, 1998; Sodek et al., 2000). These integrins are also known as receptors for RV entry into cells (Guerrero et al., 2000; Hewish et al., 2000). These pieces of evidence suggest that OPN may inhibit RV attachment or entry into cells. In addition, another study has demonstrated that OPN knockout (OPN-KO) suckling mice were more susceptible to RV infection and showed prolonged diarrhea duration compared to wild-type (WT) suckling mice (Maeno et al., 2009). On d2 post infection, mRNA levels of interleukin-1 $\beta$ , tumor necrosis factor- $\alpha$ , and interleukin-15 in OPN-KO mice were lower than in WT mice, although mRNA expression of Th-1 and Th-2 related cytokines in the small intestine were nearly the same between OPN-KO and WT mice (Maeno et al., 2009). Taken together, these findings suggested that OPN is involved in innate responses against RV infection. Innate limitations by OPN on rotavirus infection may include the elaboration by the host of protective factors that ameliorate the severity of the infection or speed mucosal repair (Rollo et al., 2005). The importance of innate responses of the host has long been recognized because children in the acute stage of typical RV infection do not develop sufficient RV-specific T cells or antibodies that are needed to overcome infections (Bhan et al., 1993; Jiang et al., 2002). Rotarix<sup>®</sup>, a live, attenuated rotavirus vaccine, mimics natural infection and stimulates robust humoral immune responses. Therefore, OPN might have no beneficial effect on antibody responses and total Igs levels to RV vaccination.

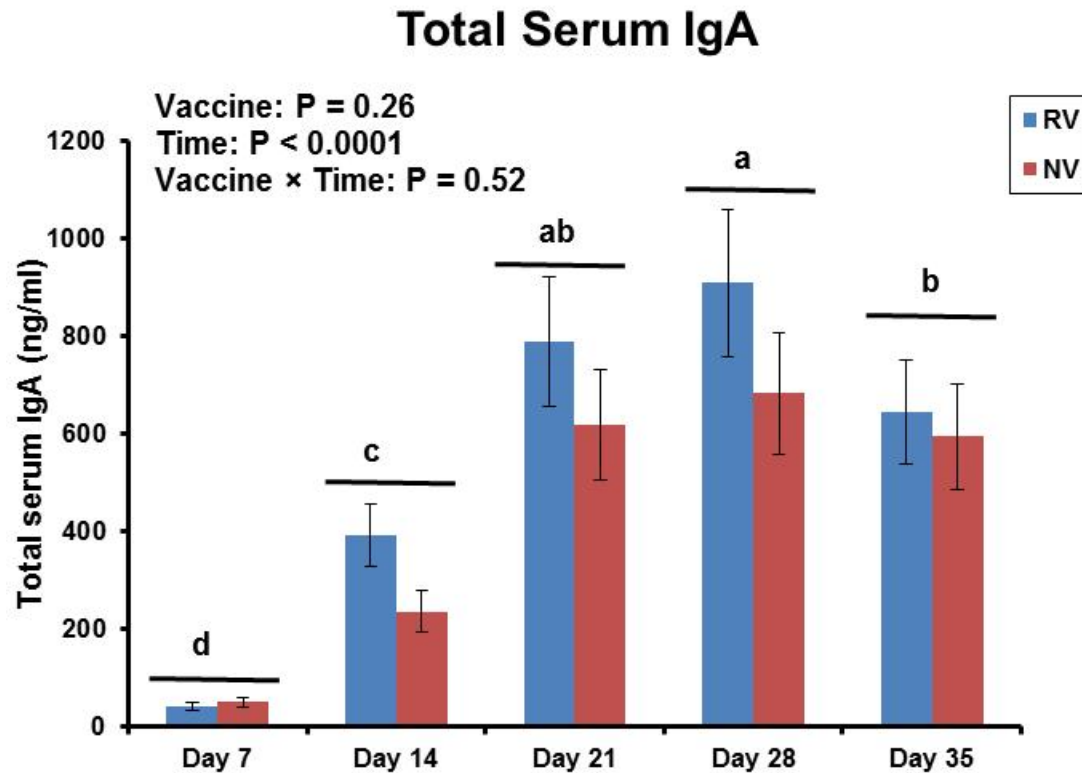
In summary, this study demonstrated that supplemental OPN did not enhance the RV-specific antibody responses (RV-specific IgG, IgA, IgM) and total Igs concentrations (total IgG, IgA, IgM) in neonatal piglets with or without RV vaccination. Since neonatal piglets generated weak antibody responses to RV vaccination, additional studies are needed to investigate the effects of OPN supplementation on immune responses to other types of vaccine that piglets will not have exposure to via colostrum.



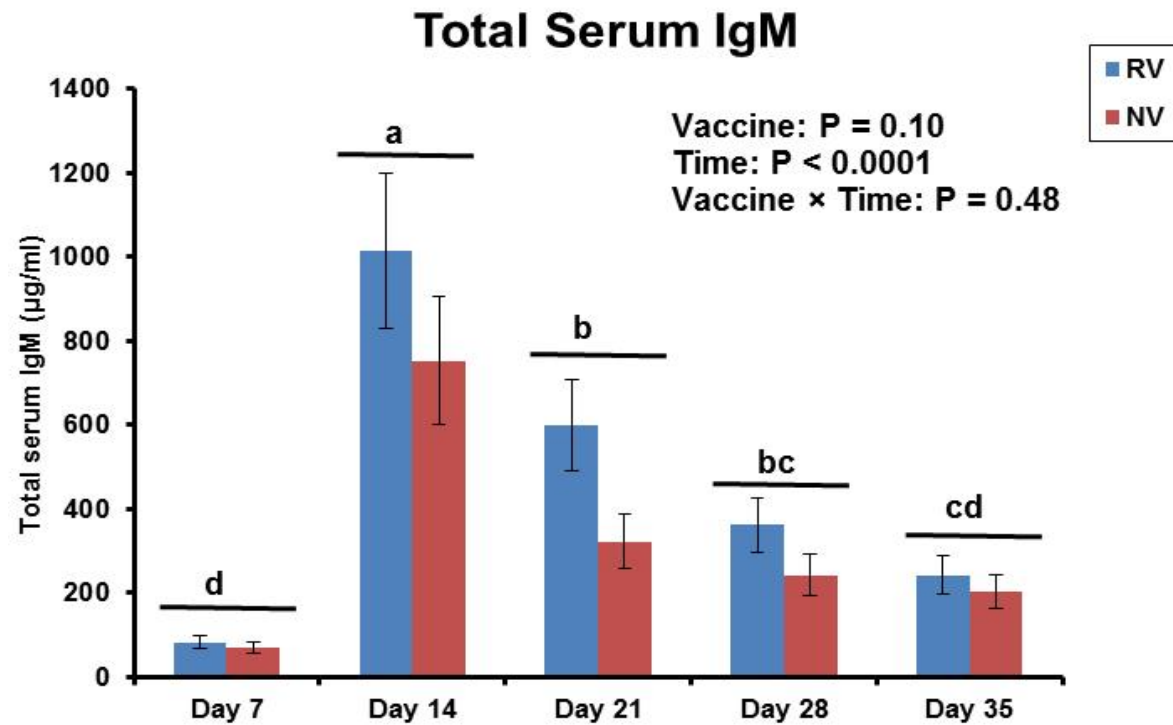
**Figure 3.1 Body weight gain.** There was no effect of OPN ( $P=0.71$ ) or vaccination ( $P=0.41$ ) on body weight gain. Since there was no dietary effect, OPN+NV and FF+NV were combined as a single non-vaccinated (NV) group. The average body weight gain for OPN+RV, FF+RV and NV was  $6.80 \pm 0.55$  kg,  $7.18 \pm 0.27$  kg and  $7.48 \pm 0.53$  kg, respectively. Values are expressed as mean  $\pm$  SEM.



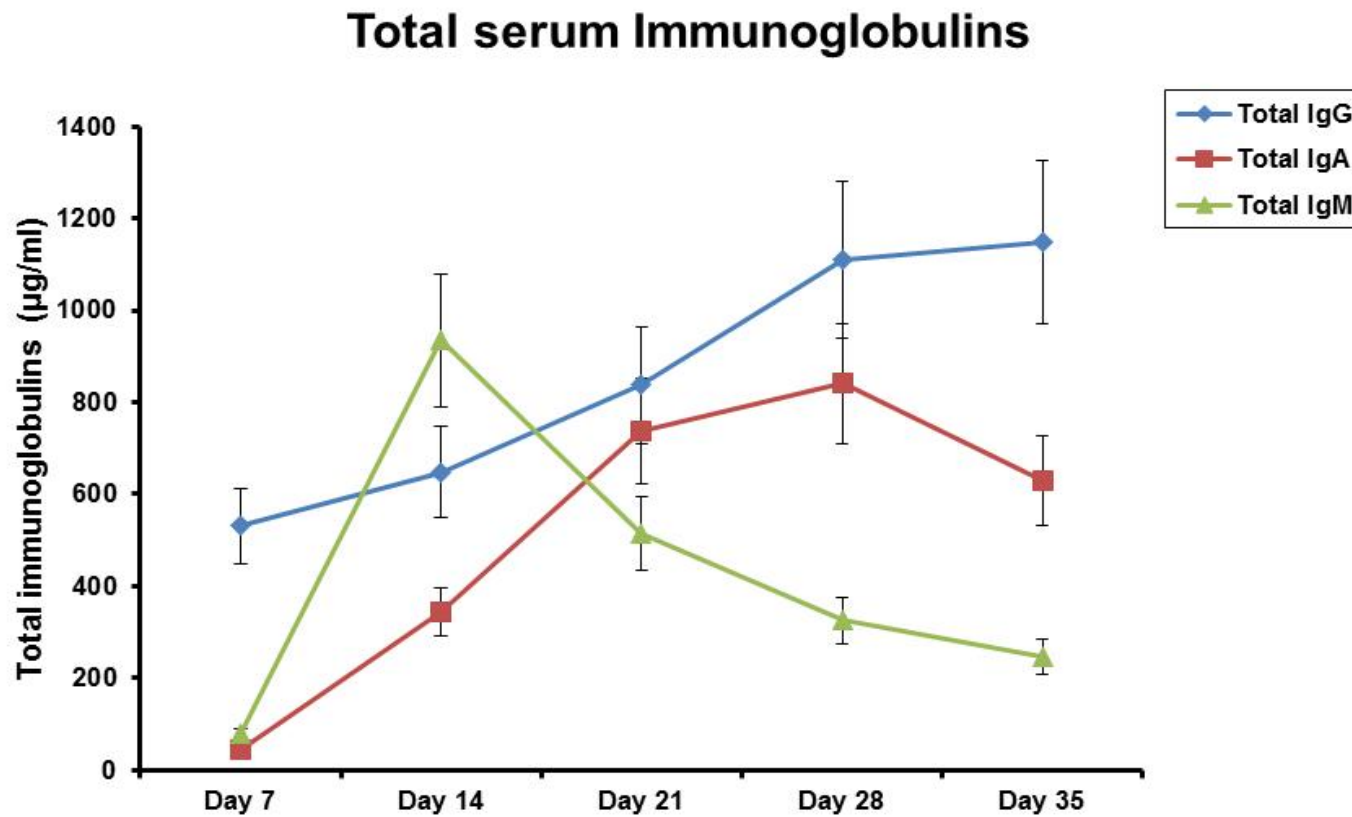
**Figure 3.2 Total serum IgG concentrations in piglets with (RV) or without RV (NV) vaccination.** Serum samples were collected on day 7, 14, 21, 28 and 35. After the combination of dietary groups, the effect of RV vaccination tended to reach statistical significance ( $P=0.05$ ). Between day 7 to day 35, total IgG concentrations in RV group were increased ( $P<0.0001$ ), ranging from  $539 \pm 107$  µg/ml (day 7) to  $1237 \pm 176$  µg/ml (day 35), while total IgG concentrations in NV group were also increased ( $P=0.0002$ ), ranging from  $511 \pm 85$  µg/ml (day 7) to  $950 \pm 158$  µg/ml (day 35). Values are expressed as mean  $\pm$  SEM. <sup>abc</sup> Values over time lacking a common letter differ at  $P<0.05$ .



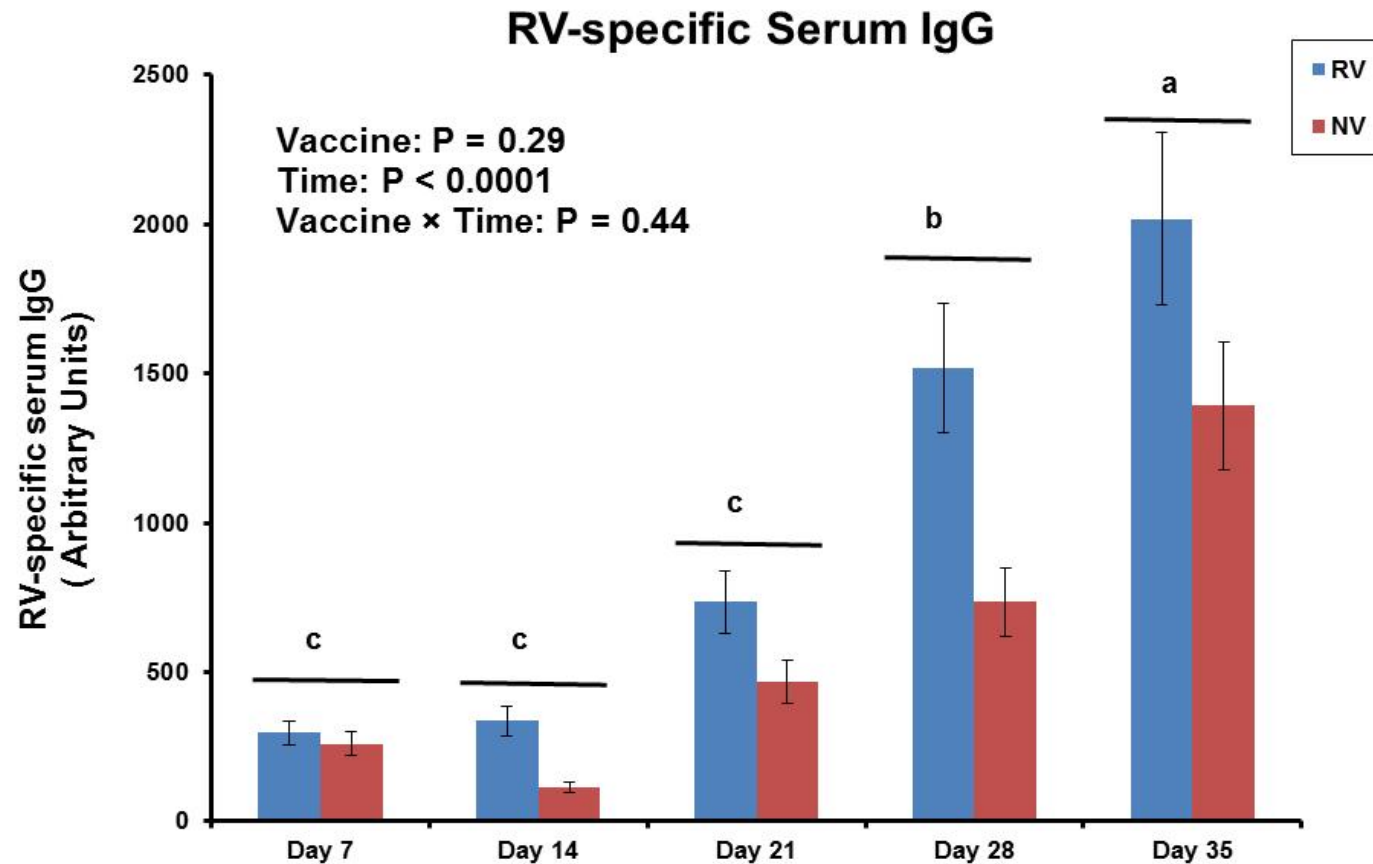
**Figure 3.3 Total serum IgA concentrations in piglets with (RV) or without RV (NV) vaccination.** Serum samples were collected on day 7, 14, 21, 28 and 35. After the combination of dietary groups, the effect of RV vaccination was still not significant ( $P=0.26$ ). Between day 7 to day 35, total IgA concentrations were changed ( $P<0.0001$ ) over time and peaked on day 28. Values are expressed as mean  $\pm$  SEM. <sup>abc</sup> Values over time lacking a common letter differ at  $P<0.05$ .



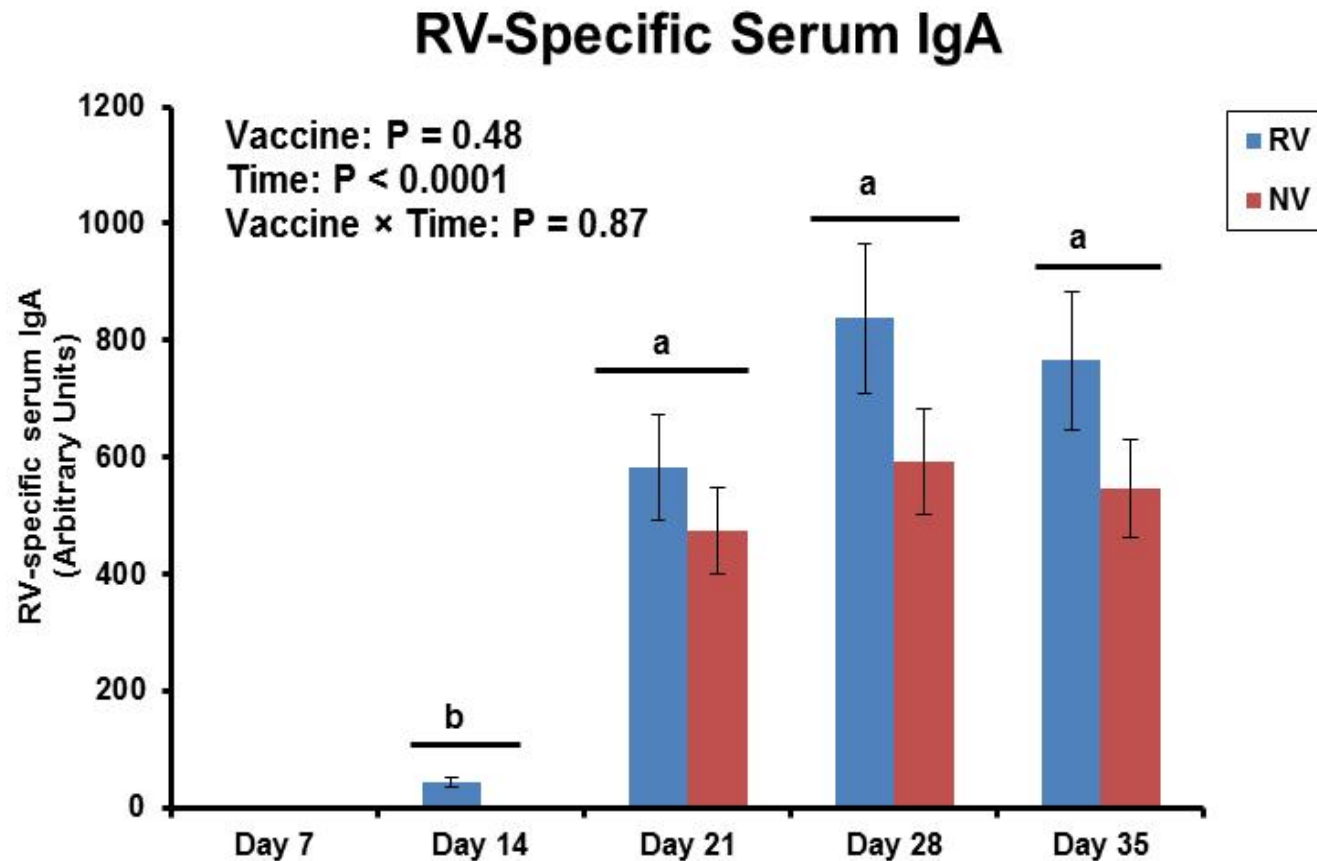
**Figure 3.4 Total serum IgM concentrations in piglets with (RV) or without RV (NV) vaccination.** Serum samples were collected on day 7, 14, 21, 28 and 35. After the combination of dietary groups, the effect of RV vaccination tended to reach statistical significance ( $P=0.10$ ). Between day 7 to day 35, total IgM concentrations were changed ( $P<0.0001$ ) over time and peaked on day 14. Values are expressed as mean  $\pm$  SEM. <sup>abc</sup> Values over time lacking a common letter differ at  $P<0.05$ .



**Figure 3.5 Total serum immunoglobulin concentrations in neonatal piglets.** Serum samples were collected on day 7, 14, 21, 28 and 35. This figure illustrates the pattern of immunoglobulins in the serum of piglets over the first 5 weeks of life.



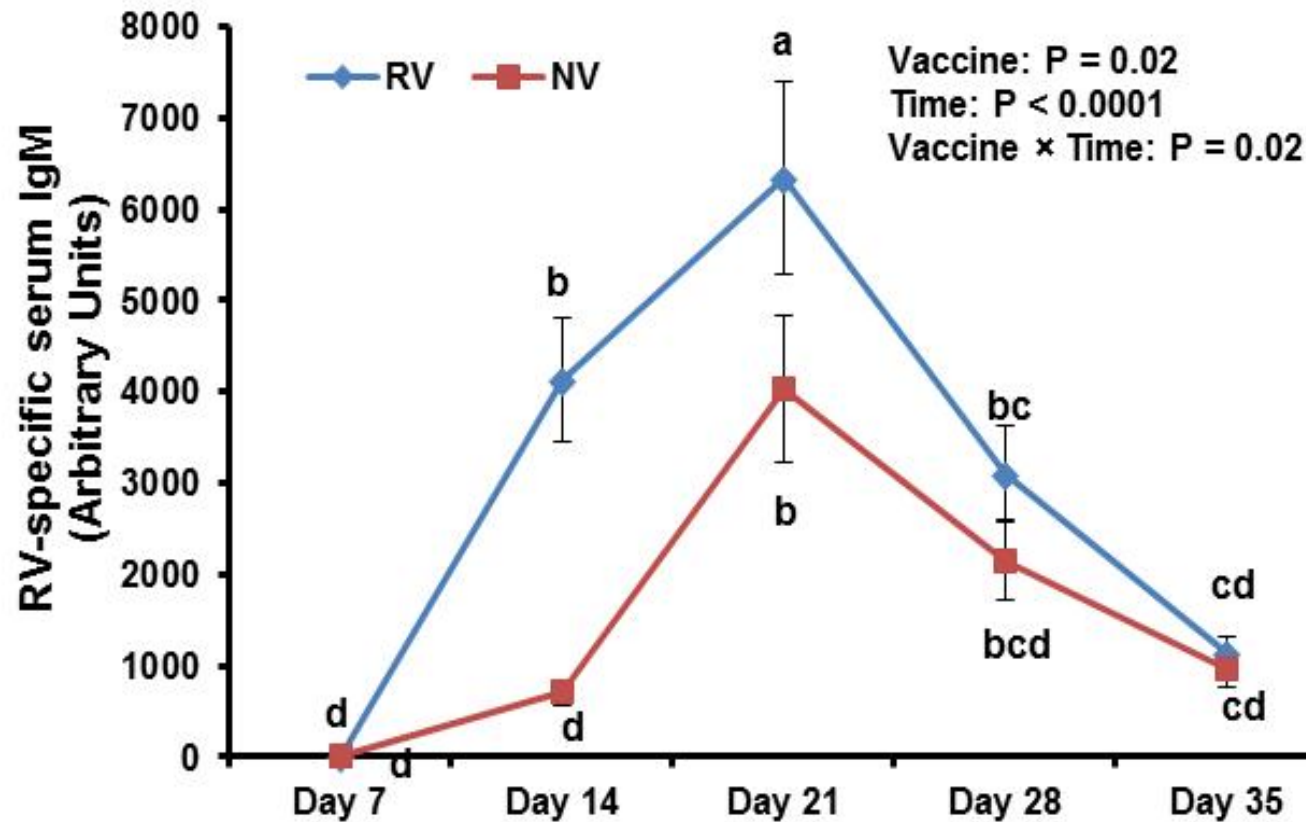
**Figure 3.6 RV-specific serum IgG concentrations in piglets with (RV) or without RV (NV) vaccination.** Serum samples were collected on day 7, 14, 21, 28 and 35. After the combination of dietary groups, the effect of RV vaccination was still not significant ( $P=0.29$ ). RV-specific IgG concentrations increased ( $P<0.0001$ ) over time. Between day 7 to day 35, RV piglets had 6.8-fold increase in RV-specific IgG, while NV piglets had 5.3-fold increase in RV-specific IgG. Values are expressed as mean  $\pm$  SEM. <sup>abc</sup> Values over time lacking a common letter differ at  $P<0.05$ .



**Figure 3.7 RV-specific serum IgA concentrations in piglets with (RV) or without RV (NV) vaccination.** Serum samples were collected on day 7, 14, 21, 28 and 35. After the combination of dietary groups, the effect of RV vaccination was still not significant ( $P=0.48$ ). All of d7 piglets had undetectable RV-specific IgA levels. RV-specific IgA concentrations were increased ( $P<0.0001$ ) over time. Values are expressed as mean  $\pm$  SEM. <sup>abc</sup> Values over time lacking a common letter differ at  $P<0.05$ .



### RV-Specific Serum IgM



**Figure 3.8 RV-specific serum IgM concentrations in piglets with (RV) or without RV (NV) vaccination.** Serum samples were collected on day 7, 14, 21, 28 and 35. After the combination of the dietary groups, the effects of RV vaccination ( $P=0.02$ ), time ( $P<0.0001$ ) and RV vaccination  $\times$  time ( $P=0.02$ ) were significant. RV-specific IgM titers were changed ( $P<0.0001$ ) over time and peaked on day 21 within each group. Values are expressed as mean  $\pm$  SEM. <sup>abc</sup> Values over time lacking a common letter differ at  $P<0.05$ .

**Table 3.1 Immunoglobulin Levels in Pregnant Sow Serum.**

**A. RV-specific immunoglobulins titers**

Arbitrary Titer	RV-Specific IgG	RV-Specific IgA	RV-Specific IgM
Sow serum	16573	58	972
Peak Antibody Titers	2018	837	6349
Ratio	8	7%	15%

**B. Total immunoglobulins concentrations**

(µg/ml)	Total IgG	Total IgA	Total IgM
Sow Serum	42904	1438	9162
Ratio	80%	2%	17%

## **Chapter 4**

### **Conclusions and Future Directions**

These studies focused on furthering our understanding of the effects of osteopontin (OPN) on intestinal development and serum antibody responses to rotavirus (RV) vaccination in neonates. In Chapter 2, piglet intestinal development was evaluated by measuring intestinal weight and length, mucosal protein and DNA content, disaccharidase activity, villus morphology and jejunal enterocyte proliferation. Overall, OPN supplementation in formula exerts minor effects within the small intestine. Bovine OPN added formula at human milk concentrations did not appear to significantly affect intestinal structural and functional development in neonatal piglets.

The biological activity of a purified protein is an important consideration when assessing its biological function. In milk, OPN is present as an intact protein or in several proteolytically generated N- and C-terminal fragments (Sørensen and Petersen, 1993b; Sørensen et al., 2003). Since there is currently no antibody against porcine OPN available, we were unable to measure the concentration of OPN in either sow milk or piglet serum. Thus, the OPN intake of the SR piglets from sow milk is unknown. In addition, whether serum OPN concentrations were higher in OPN compared to FF piglets could not be assessed. However, OPN purified from bovine milk was shown to induce IL-12 expression in cultured human intestinal mononuclear cells (Schack et al. 2009). This is further supported by the observation that bovine milk OPN was partially resistant to proteolysis by neonatal gastric juice (Chatterton et al., 2004). These studies suggest that the purified protein retains its bioactivity; however, further studies are needed to determine

whether supplemental bovine OPN survives digestion *in vivo* and is absorbed into the piglet circulation.

Previous work from our laboratory has shown that the supplementation of OPN to formula at human milk concentrations decreased the differences in jejunal gene expression between formula-fed and mother-fed Rhesus monkeys (Donovan et al., 2011). Differences in signaling pathways regulating stem cell proliferation (WNT), gastrointestinal patterning (HEDGEHOG) and cell fate between secretory and absorptive lineages (NOTCH) were differentially expressed among the groups. Consequently, future work could identify gene expression in the jejunum of these piglets using gene microarray to determine whether supplemental OPN is also modulating the intestinal transcriptome of piglets.

The objective of Chapter 3 was to investigate whether supplemental OPN influenced the serum antibody responses to rotavirus vaccination (Rotarix<sup>®</sup>, a live attenuated rotavirus vaccine, mimics natural infection and stimulates humoral immune responses.) in neonatal piglets. Our results suggested that supplemental OPN had no effect on RV-specific serum antibody responses and total serum Ig levels in neonatal piglets. This may alternatively support previous findings that OPN is involved in innate responses against RV infection. Innate limitations by OPN on RV infection may include the inhibition of RV attachment or entry into cells or regulation of epithelial restitution (Rollo et al., 2005). A recent study has shown that ingested OPN decreased necrotising enterocolitis (NEC) severity (Møller et al., 2011). Therefore, the potential mechanisms of innate immune function of OPN against RV vaccination or infection remains to be elucidated in future studies.

Overall, our piglet model (colostrum-deprived, orally serum-gavaged) generated weak antibody response to RV vaccination. Many reports indicate that RV-specific serum

immunoglobulins (Igs), especially IgA, were a good marker of protection against RV infection and moderate-to-severe diarrhea (Velazquez et al., 2000; Parreño et al., 1999). However, robust RV-specific IgA response was not observed in our study. Intestinal IgA antibody and fecal IgA antibody are better correlates of protection from RV infection (Brown et al., 2000). Large-size animal studies are needed to extend our results by measuring RV-specific IgA, IgG, IgM concentrations in intestinal fluid or feces. To evaluate the effector B cell and memory B cell responses, ELISPOT assays could be used to enumerate RV-specific antibody secreting cell (ASC) of different isotypes (IgA, IgG and IgM).

In addition, the weak antibody responses to RV vaccination suggested that the sow serum with maternal antibodies used in our study, although protecting piglets against environmental pathogens, may impair the development of active immunity and decrease the efficacy of live oral rotavirus vaccines. To further investigate the immunological roles of OPN, an influenza (Fluzone<sup>TM</sup>) vaccination piglet study with OPN supplementation could be conducted to determine the effects of OPN in the development of vaccine-induced antibody responses. Previous work from our laboratory demonstrated that Fluzone<sup>TM</sup> vaccination induced robust antibody responses. Therefore, it is possible to assess the role of bovine OPN in the development of infants' immune responses to influenza vaccination.

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## Appendix A: Supplemental Tables

**Table A.1. Effect of Dietary Treatment and Vaccination on Total Serum IgG.<sup>1,2,3</sup>**

	<b>Day 7</b>	<b>Day 14</b>	<b>Day 21</b>	<b>Day 28</b>	<b>Day 35</b>
<b>FF + RV</b>	542 ± 83	678 ± 104	942 ± 145	1359 ± 209	1396 ± 215
<b>OPN + RV</b>	536 ± 75	762 ± 109	985 ± 140	1147 ± 164	1109 ± 158
<b>FF + NV</b>	557 ± 92	407 ± 66	581 ± 95	880 ± 147	951 ± 158
<b>OPN + NV</b>	466 ± 77	535 ± 89	509 ± 84	755 ± 126	949 ± 158

<sup>1</sup> Data are expressed as mean ± SEM

<sup>2</sup> Abbreviations: FF, formula-fed; OPN, formula with OPN; NV, non-vaccinated; V, vaccinated with Rotarix<sup>®</sup>

<sup>3</sup> In the complete model, no effect of OPN (P=0.71), RV vaccination (P=0.07) or OPN × RV vaccination (P=0.88) was observed. The only significant effect is time (P=0.0006).

**Table A.2. Effect of Dietary Treatment and Vaccination on Total Serum IgA.**<sup>1,2,3</sup>

	<b>Day 7</b>	<b>Day 14</b>	<b>Day 21</b>	<b>Day 28</b>	<b>Day 35</b>
<b>FF + RV</b>	33 ± 4	496 ± 70	753 ± 107	912 ± 130	670 ± 93
<b>OPN + RV</b>	47 ± 7	309 ± 45	818 ± 120	908 ± 133	624 ± 91
<b>FF + NV</b>	49 ± 9	235 ± 42	588 ± 106	701 ± 127	636 ± 115
<b>OPN + NV</b>	51 ± 8	236 ± 43	651 ± 118	666 ± 121	554 ± 101

<sup>1</sup> Data are expressed as mean ± SEM

<sup>2</sup> Abbreviations: FF, formula-fed; OPN, formula with OPN; NV, non-vaccinated; V, vaccinated with Rotarix<sup>®</sup>

<sup>3</sup> In the complete model, no effect of OPN (P=0.85), RV vaccination (P=0.31) or OPN × RV vaccination (P=0.92) was observed. The only significant effect is time (P<0.0001).

**Table A.3. Effect of Dietary Treatment and Vaccination on Total Serum IgM.**<sup>1,2,3</sup>

	<b>Day 7</b>	<b>Day 14</b>	<b>Day 21</b>	<b>Day 28</b>	<b>Day 35</b>
<b>FF + RV</b>	81 ± 11	903 ± 129	487 ± 69	361 ± 51	265 ± 38
<b>OPN + RV</b>	84 ± 11	1105 ± 162	605 ± 89	395 ± 58	253 ± 38
<b>FF + NV</b>	71 ± 10	779 ± 120	287 ± 44	270 ± 54	284 ± 43
<b>OPN + NV</b>	68 ± 10	728 ± 112	360 ± 55	216 ± 21	221 ± 34

<sup>1</sup> Data are expressed as mean ± SEM

<sup>2</sup> Abbreviations: FF, formula-fed; OPN, formula with OPN; NV, non-vaccinated; V, vaccinated with Rotarix<sup>®</sup>

<sup>3</sup> In the complete model, no effect of OPN (P=0.74), RV vaccination (P=0.13) or OPN × RV vaccination (P=0.33) was observed. The only significant effect is time (P<0.0001).

**Table A.4. Effect of Dietary Treatment and Vaccination on Rotavirus-Specific Serum IgG.<sup>1,2,3</sup>**

	<b>Day 7</b>	<b>Day 14</b>	<b>Day 21</b>	<b>Day 28</b>	<b>Day 35</b>
<b>FF + RV</b>	290 ± 41	320 ± 46	567 ± 81	1620 ± 231	1870 ± 267
<b>OPN + RV</b>	300 ± 46	349 ± 54	867 ± 133	1453 ± 224	2136 ± 329
<b>FF + NV</b>	275 ± 39	104 ± 15	443 ± 63	549 ± 78	1356 ± 194
<b>OPN + NV</b>	245 ± 33	126 ± 17	492 ± 68	919 ± 130	1428 ± 202

<sup>1</sup> Data are expressed as mean ± SEM

<sup>2</sup> Abbreviations: FF, formula-fed; OPN, formula with OPN; NV, non-vaccinated; V, vaccinated with Rotarix<sup>®</sup>

<sup>3</sup> In the complete model, no effect of OPN (P=0.82), RV vaccination (P=0.35) or OPN × RV vaccination (P=0.99) was observed. The only significant effect is time (P<0.0001).

**Table A.5. Effect of Dietary Treatment and Vaccination on Rotavirus-Specific Serum IgA.**<sup>1,2,3</sup>

	<b>Day 7</b>	<b>Day 14</b>	<b>Day 21</b>	<b>Day 28</b>	<b>Day 35</b>
<b>FF + RV</b>	0	47 ± 8	406 ± 68	829 ± 138	673 ± 112
<b>OPN + RV</b>	0	42 ± 7	561 ± 89	844 ± 134	840 ± 131
<b>FF + NV</b>	0	0	402 ± 62	603 ± 92	523 ± 80
<b>OPN + NV</b>	0	0	545 ± 84	582 ± 89	571 ± 88

<sup>1</sup> Data are expressed as mean ± SEM

<sup>2</sup> Abbreviations: FF, formula-fed; OPN, formula with OPN; NV, non-vaccinated; V, vaccinated with Rotarix<sup>®</sup>

<sup>3</sup> In the complete model, no effect of OPN (P=0.76), RV vaccination (P=0.54) or OPN × RV vaccination (P=0.92) was observed. The only significant effect is time (P=0.0004).

**Table A.6. Effect of Dietary Treatment and Vaccination on Rotavirus-Specific Serum IgM.**<sup>1,2,3</sup>

	<b>Day 7</b>	<b>Day 14</b>	<b>Day 21</b>	<b>Day 28</b>	<b>Day 35</b>
<b>FF + RV</b>	0	5117 ± 852	8355 ± 1390	3922 ± 653	1273 ± 212
<b>OPN + RV</b>	3 ± 0.7	5139 ± 790	7983 ± 1228	4254 ± 654	1569 ± 241
<b>FF + NV</b>	0	608 ± 103	3941 ± 668	3186 ± 540	1059 ± 179
<b>OPN + NV</b>	0	808 ± 136	4136 ± 701	1108 ± 187	853 ± 144

<sup>1</sup> Data are expressed as mean ± SEM

<sup>2</sup> Abbreviations: FF, formula-fed; OPN, formula with OPN; NV, non-vaccinated; V, vaccinated with Rotarix<sup>®</sup>

<sup>3</sup> In the complete model, no effect of OPN (P=0.98), RV vaccination (P=0.04) or OPN × RV vaccination (P=0.56) was observed. The only significant effect is time (P<0.0001).

# CURRICULUM VITAE

## Jin Lu

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## EDUCATION

### University of Illinois at Urbana-Champaign (UIUC)

Master of Science in Nutritional Sciences, August 2011

Thesis Title: Effects of supplemental osteopontin on intestinal development and serum antibody responses to rotavirus vaccination in piglets

Thesis Advisor: Professor Sharon Donovan

### Tianjin Medical University (TMU)

Bachelor of Science in Preventive Medicine, 2009

Thesis Title: Analysis on the relations between hyperuricemia and its dietary risk factors in health survey population

Thesis Advisor: Professor Hong Chang

## RESEARCH EXPERIENCE

### 08.2009–current Graduate Research Assistant, Division of Nutritional Sciences, UIUC

Research advisor: Professor Sharon Donovan

- i. Developed rotavirus-specific immunoglobulin(Ig) G, IgM and IgA enzyme-linked immunosorbent assay(ELISA) and total IgG, IgA and IgM ELISA;
- ii. Measured villous height and crypt depth in the duodenum, jejunum and ileum by histomorphology;
- iii. Measured lactase and sucrase activities in the duodenum, jejunum and ileum by disaccharidase assay;
- iv. Measured protein concentration of the intestinal mucosa homogenate by bradford protein assay;
- v. Isolated mononuclear cells from peripheral blood, mesenteric lymph nodes and spleen;

### 08. 2007-07.2009 Undergraduate Student, School of Public Health, TMU

Research advisor: Professor Guowei Huang

- i. Measured plasma homocysteine by high-performance liquid chromatography (HPLC);
- ii. Practiced the establishment of a rat model of focal cerebral ischemia through middle cerebral artery occlusion (MCAO);
- iii. Conducted multivariate logistic regression analysis by SPSS.

## **PUBLICATIONS AND PRESENTATION**

### **Journal papers**

- i. **Lu J.**, Huang G., Chang H., Zhang X., Liu H. Effects of folic acid on plasma homocysteine level of rats with cerebral ischemia. *Tianjin Medical Journal*. 37(1): 61-64, 2009.

### **Poster presentation**

**Lu J**, Shunk JM, Comstock SS, Monaco MH, Donovan SM. "Detection of rotavirus specific immunoglobulins in the serum of neonatal piglets with or without colostrum feeding." Experimental Biology, Washington, DC, April 9-13, 2011.

## **WORK EXPERIENCE (INTERNSHIP)**

- i. **10.2008-11.2008 Center of Disease Control, Tianjin, China**  
Analyzed data relevant to public health and medicine by means of **SAS, SPSS**
- ii. **09.2008-10.2008 Nutrition Department, Tianjin Third Central Hospital, Tianjin, China**  
Learned the functions and preparation of the parenteral and enteral nutrient formulas for patients
- iii. **05.2007-06.2007 Department of Obesity, Metabolic Diseases Hospital, Tianjin, China**  
Learned diagnosis, treatment and prevention of diabetes and obesity  
**04.2006-06.2007 Tianjin Machang Community School, Tianjin, China**  
Gave public presentations on "Nutritious Components in Food"

## **HONORS AND AWARDS**

- |                    |   |
|--------------------|---|
| <b>2011</b>        | Certificate in Business Administration, UIUC        |
| <b>2011</b>        | Toshiro Nishida Research Award, UIUC                |
| <b>2011</b>        | Abbott Nutrition Scholarship, UIUC                  |
| <b>2009 – 2011</b> | Graduate Research Assistantship, UIUC               |
| <b>2006 – 2008</b> | University Scholarship for Academic Excellence, TMU |

## **PROFESSIONAL ACTIVITIES**

- |                  |  |
|------------------|--|
| <b>2010-2011</b> | Student member of American Society for Nutrition                                 |
| <b>04.2010</b>   | Volunteer for Annual Nutrition Symposium, Division of Nutritional Sciences, UIUC |
| <b>11.2009</b>   | Volunteer for Quiz Bowl, Division of Nutritional Sciences, UIUC                  |
| <b>08.2008</b>   | Volunteer for 2008 Beijing Olympic Games   |